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Interaction of Myo-inositol 1,4,5-trisphosphate with its receptor and metabolic enzymes in platelets: Modulation by structural analogues

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Interaction of *Myo*-inositol 1,4,5-trisphosphate with its receptor and metabolic enzymes in platelets: Modulation by structural analogues.

Submitted by Jenan Al-Hafidh

for the degree of

Doctor in Philosophy

at the University of Bath

1994

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ABSTRACT.

Myo-inositol 1,4,5-trisphosphate-induced Ca^{2+} -release from permeabilised platelets was found to be stereospecific. D-InsP₃ was found to be a full agonist whereas its enantiomer, *L-my*o-inositol 1,4,5-trisphosphate was inactive. A temperature dependence of InsP₃-induced Ca^{2+} -release was also established. Parallel shift of the InsP₃-dose response curve to the right were observed with increasing temperatures from 4°C to 37°C. This reduction in apparent efficacy was mirrored by the more rapid rate of InsP₃ metabolism. InsP₃ is metabolised faster at 37°C than at 4°C, and the apparent decrease in potency of InsP₃ with the increase in temperature may be due to this increased metabolism.

With the aid of the two inositol phosphate analogues, *L-my*o-inositol 1,4,5-trisphosphorothioate and *my*o-inositol 1,3,5-trisphosphorothioate, we have confirmed the presence of different forms of the 5-phosphatase enzyme in platelets. InsP₃ selective 5-phosphatase and an Ins(1,3,4,5)P₄ selective 5-phosphatase have been demonstrated.

A structure-activity relationship has been established for the structural requirements for InsP₃-induced Ca^{2+} -mobilisation in platelets at the InsP₃ receptor. It is apparent that the vicinal 4,5-bisphosphate is essential for activity as shown when examining analogues with modifications at these positions. The hydroxyls on the 2-, 3- and 6-positions play a less considerable role, with only the 6-hydroxyl playing a small, but significant role in receptor activation and Ca^{2+} -mobilisation.

As a result of examining over 30 inositol phosphate analogues, to date, the best receptor antagonists uncovered are the three partial agonists *scyllo*-inositol 1,2,4,5-tetrakisphosphorothioate, *L-chiro*-inositol 2,3,5-trisphosphorothioate and *my*o-

inositol 1,4,6-trisphosphorothioate. These will provide tools in designing future antagonist.

Studies on the role of Ca^{2+} -entry upon InsP_3 -production were investigated using the Ca^{2+} -influx inhibitor SKF 96365. However, this compound was not found to be a pure influx inhibitor in platelets, but that it was capable of inhibiting PAF-induced InsP_3 -production, 5-HT release and Ca^{2+} -mobilisation. It was, however, unable to modify InsP_3 - and $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Ca^{2+} -release.

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ABBREVIATIONS

Inositol lipids and phosphates

PI	Phosphoinositide
PtdIns	Phosphatidylinositol
PtdIns(3)P	Phosphatidylinositol 3-phosphate
PtdIns(4)P	Phosphatidylinositol 4-phosphate
PtdIns(3,4)P	Phosphatidylinositol 3,4-bisphosphate
PtdIns(4,5)P	Phosphatidylinositol 4,5-bisphosphate
PtdIns(3,4,5)P	Phosphatidylinositol 3,4,5-trisphosphate

All the inositol phosphates are based upon *myo*-inositol, unless otherwise stated. All inositol phosphates refer to the D-isomer, unless stated. The positions of the phosphates around the inositol ring are given in parentheses to indicate a specific analogue. Abbreviations of the structures and names of all the analogues examined can be found in appendix I.

Other abbreviations.

AA	arachidonic acid
AC	adenylate cyclase
ADP	adenosine 5'-diphosphate
ADP-ribose	adenosine diphosphate ribose
ATP	adenosine 5'-trisphosphate
BAPTA	1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid.
BSA	bovine serum albumin
cAMP	adenosine 3':5'-cyclic monophosphate
cADP-ribose	cyclic adenosine diphosphate ribose

$[Ca^{2+}]_e$	extracellular calcium concentration
$[Ca^{2+}]_i$	intracellular calcium concentration
$CHCl_3$	chloroform
CPM	counts per minute
DAG	diacylglycerol
DMSO	dimethylsulphoxide
EC ₅₀	concentration producing 50% of maximal response
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis(B-aminoethylether) N,N,N',N'-tetraacetic acid
Fura-2 AM	fura-2 acetomethoxy ester
G-protein	guanine nucleotide-binding proteins
G _i	G-protein associated with inhibition of AC
G _s	G-protein associated with activation of AC
HBT	hepes buffered tyrode
5-HT	5-hydroxytryptamine
HPLC	high pressure liquid chromatography
IC ₅₀	concentration producing 50% inhibition of response
IC ₅₀	concentration of agent inhibiting a response by 50%
ICB	"intracellular" like buffer
LT's	leukotrienes
NO	nitric oxide
PAF	platelet activating factor
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PGL ₂	prostacyclin
PIP ₂	PtdIns(4,5)P ₂

PKC	protein kinase C
PLA₂	phospholipase A ₂
PLC	phospholipase C
PRP	platelet rich plasma
RMCE	receptor mediated calcium entry
TXA₂	thromboxane
vWF	von Willebrands factor

INTRODUCTION

Overview

Rabbit platelets were used to study phosphoinositide (PI) signalling. Platelets provide an ideal model for studying the effects of D-*myo*-inositol 1,4,5-trisphosphate (InsP₃) and its analogues as they do not possess a nucleus or RNA that will complicate and interfere with the signalling mechanism. This thesis is written in such a way that the introduction contains a literature review of research carried out until the start of this study, i.e. until 1990. The rest of the literature is reviewed, along with my results, in the discussion section.

1 The blood platelet.

Blood platelets circulate as smooth anucleate biconvex discs, 1 μ m thick and 2-3 μ m in diameter. They are formed by fragmentation of the cytoplasm of megakaryocytes in the bone marrow. In general, platelets are incapable of protein synthesis in biologically significant quantities, however newly formed platelets often contain ribosomes and RNA from the parent cell cytoplasm. The platelet count in peripheral blood is 2.5×10^8 /ml with a turnover of 3×10^7 /ml/day. The half life of the platelet in circulation is 8- 10 days, for review see Siess, (1989). Although platelets contain no nucleus, they contain cellular organelles such as mitochondria, open canilicular systems, microtubules, dense tubular system and storage granules (dense granules, α_1 granules and α_2 granules) (Crawford and Scrutton, 1987).

The major purpose of platelets in the circulation is participation in the haemostatic reaction, where they play a role in the formation of a cellular plug at the site of vascular damage fulfilling their primary role in arresting bleeding.

Although most of our understanding of blood platelets concerns the role of this cell in haemostasis and in disorders of this process such as thrombosis, the ease of isolation of a homogeneous cell population, the lack of a nucleus to complicate cellular responses and the wide range of functions platelets possess has meant that they have been used extensively to study signal transduction, adhesion and secretion.

1.1 The basic platelet response.

Platelets are very rapidly activated and respond to a wide range of agonists upon cell surface receptor occupation. The platelet response can be classified into two categories that may be related to different functions of platelets *in vivo*. First, the reversible platelet response, including adhesion, shape change and primary reversible aggregation and second, the irreversible platelet response that comprises release reaction and secondary irreversible aggregation. These latter irreversible responses are involved in the haemostatic function of platelets reviewed in Siess, (1989).

Resting circulating platelets are discoid and have a smooth ripple surface. The first physiological responses upon stimulation are platelet adhesion and shape change. Physiological vasodilation, which leads to gaps within the endothelial lining or pathophysiological vascular injury resulting in the exposure of subendothelial tissue. Both these effects contribute to the adhesion process. Platelets adhere to the subendothelial material by their interaction with two specific macromolecule proteins, collagen and von Willebrand's factor (vWf). Adhesion proceeds in two distinct phases: (i) an initial contact phase in which a platelet is bound to the vessel wall by a small amount of membrane or through pseudopodia and (ii) a spreading phase in which the platelet is more intimately

bound to the subendothelium by a large amount of endothelium (Leytin *et al.*, 1984).

Following adhesion, shape change occurs. This is characterised by two distinct morphological features; the spheration of the platelet and the extrusion of pseudopods. In addition, the membrane surface becomes irregular and folded. All platelet stimuli except adrenaline and phorbol esters induce full platelet shape change. Adrenaline and phorbol esters do not raise $[Ca^{2+}]_i$, inducing only some pseudopod formation without platelet spheration (Bendtzen *et al.*, 1983; Cusack and Hourani, 1982). This indicates that platelet spheration and pseudopod formation are separate independent events.

Subsequent to adhesion and shape change, aggregation can occur. *In vitro* this can be distinguished into two types, primary and secondary aggregation. The former is reversible and can occur without a release reaction and the latter is irreversible and associated with the release reaction. Primary reversible aggregation is induced by low concentrations of platelet stimuli in the presence of extracellular Mg^{2+} or Ca^{2+} . Primary aggregation results from the exposure of the fibrinogen receptor and some subtle change in the glycoproteins IIb and IIIa giving rise to the formation of reversible cross-linking of platelets by fibrinogen molecules (Bennett and Vilaire, 1979). Secondary aggregation results from reinforcing these fibrinogen links by secreted thrombospondin (Leung and Nachman, 1982).

The final step is the release reaction (Holmsen *et al.*, 1969; Holmsen, 1978; Holmsen, 1985). This occurs by the exocytotic discharge of the granule contents. There are at least three types of secretory granules; dense-, α_1 - and α_2 (lysosomal) secretory granules. These three storage granules can be

distinguished by their density and their discharge is under differential or graded control (Thevenod *et al.*, 1989).

In general a lower concentration of agonist is needed to release the contents of the α_1 -granules than the dense granules (Holmsen, 1978), for review see Siess (1989).

The platelet haemostatic function must be highly regulated in order to allow the formation of a haemostatic plug when needed and otherwise prevent haemorrhage or thrombosis. The balance between antiaggregatory and proaggregatory signals is achieved by compounds which are able to promote or inhibit platelet activation. These compounds originate from an extrinsic source (thrombin and vWf), from activated platelets themselves [adenosine diphosphate (ADP), 5-HT, Ca^{2+} , thromboxane (TxA_2)] or from the vascular endothelium [PGI_2 , nitric oxide (NO)] (Siess, 1989). The release reaction has a self-amplification effect on the response where the release of the granule contents such as ADP and 5-HT stimulate the activation of yet other platelets, thus amplifying the original stimulus.

The vascular endothelium also plays a role in haemostasis. It is capable of producing NO and this causes an increase in cGMP levels by acting through a soluble guanylate cyclase. In platelets, an increase in levels of cGMP is anti-aggregatory, inhibiting aggregation (Radomski *et al.*, 1990; Siess, 1989).

Furthermore PGI_2 , which is also released from the vascular endothelium, also acts to inhibit aggregation (McIntyre *et al* 1985) by activating adenylate cyclase and increasing cAMP levels (Siess, 1989).

Two major pathways have been proposed for AA liberation (see figure 1); a direct deacylation of membrane phospholipids by PLA_2 or

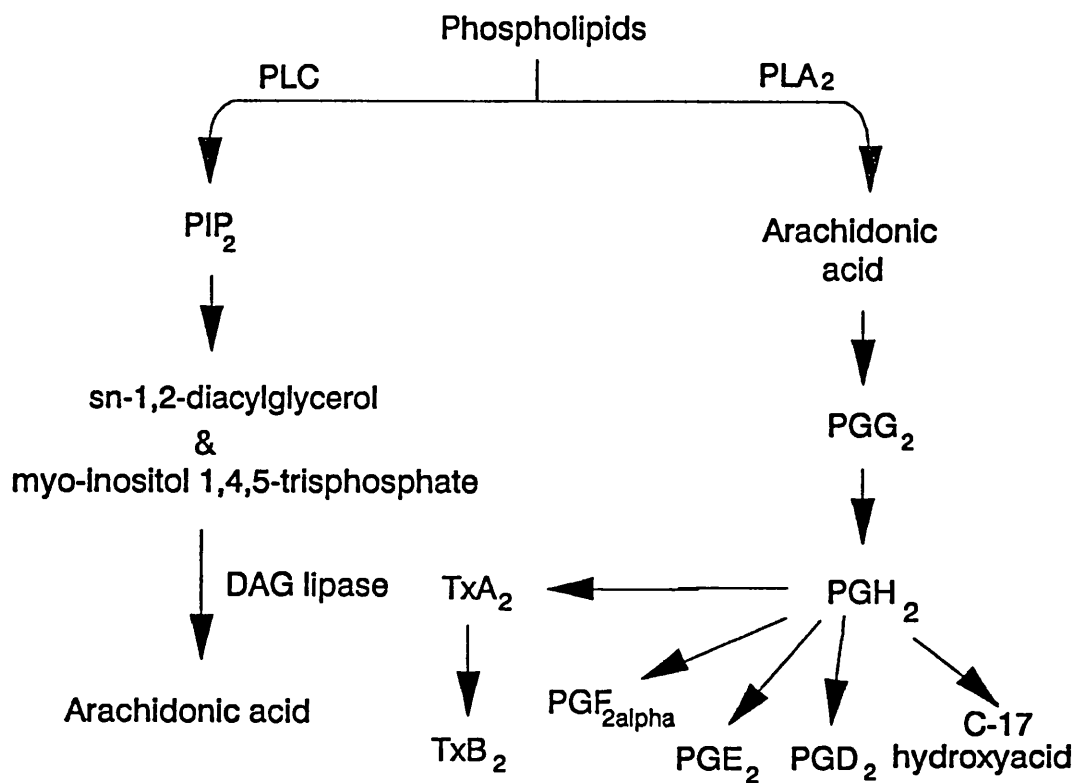


Figure 1. The biosynthetic pathway for arachidonic acid (AA) formation and its metabolites through the cyclo-oxygenase pathway.

the sequential action of PLC on phosphatidylinositol lipids followed by the action of DAG-lipase, the former route having a greater role. Ca^{2+} dependent activation of PLA_2 and subsequent eicosanoid biosynthesis is considered essential to promote platelet self-amplification (Siess, 1989; Lapetina *et al.*, 1986). TxA_2 is a potent vasoconstrictor and agonist on platelets.

Its action is short lived due to its rapid metabolism to TxB_2 (Hamberg *et al.*, 1975) PLA_2 is an enzyme involved in the liberation from membrane phospholipids of an arachidonyl residue (AA) from membrane phospholipids which can be subsequently metabolized to various bioactive lipid mediators such as PGs, TxA_2 and leukotrienes (Takayama *et al.*, 1991). The isolation of a cytosolic soluble PLA_2 from rabbit platelets has recently been implicated in AA liberation in rabbit stimulated platelets (Takayama *et al.*, 1991).

1.2 Platelet agonists and their signalling mechanisms.

Platelets are non-electrically excitable cells (Thevenod *et al.*, 1989). The absence of a nucleus and hence nuclear receptors means that activation is confined to cell surface receptor activation. Thus, surface receptors have coupling mechanisms which allow a signal to be carried (transduced) from the cell surface receptor through the membrane to the cytosol, leading to the formation or liberation of intracellular messenger molecules. There are several components of signal transducing mechanisms these include; D-*myo*-inositol 1,4,5-trisphosphate (InsP_3), Ca^{2+} , cAMP, diacylglycerol (DAG) and protein tyrosine kinases (PTK's) (Berridge and Irvine, 1989; Berridge, 1986) which in turn act on targets inside the cell.

Many different agents can activate the same series of responses in platelets.

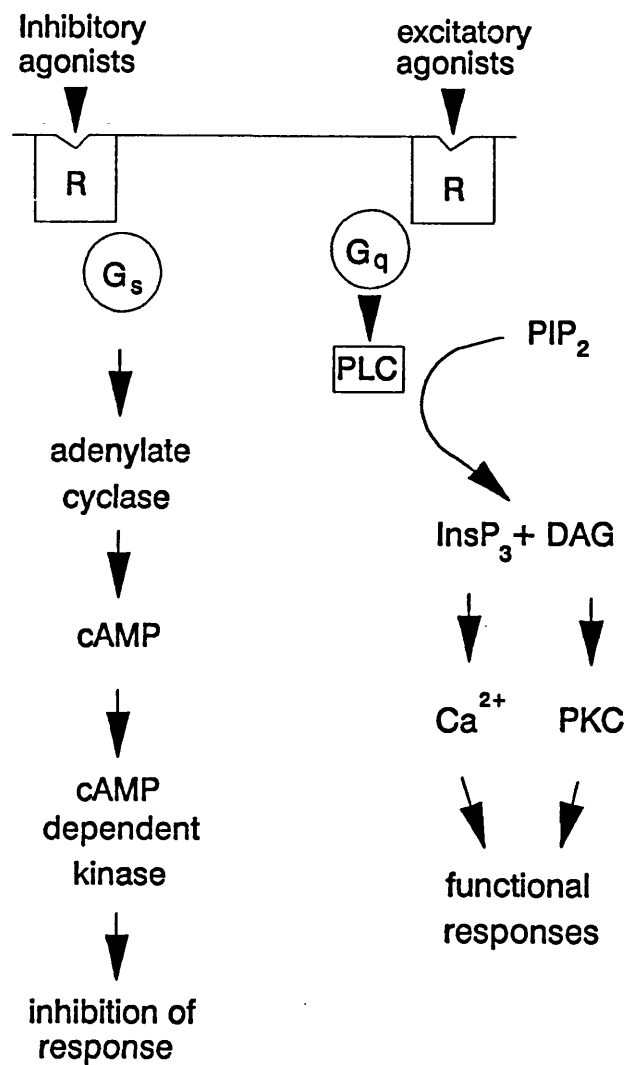


Figure 2. The signalling mechanisms of excitatory and inhibitory platelet agonists.

These responses are achieved by the interaction of each of these agonists with specific cell surface receptors, based upon demonstration of saturable binding and the existence of the same specific inhibitors of both binding and platelet response. Thus, while there is a high degree of specificity at the level of the agonist-receptor interaction, this specificity is almost lost by the appearance of the same final responses, (Holmsen, 1985). The specificity that remains at the level of response execution is of a quantitative nature. These platelet agonists can thus be divided into weak, intermediately strong and strong agonists according to how far in the response sequence they drive the platelets.

All platelet agonists trigger PIP₂ hydrolysis, with the exception of adrenaline and collagen, see figure 2. Thus, agonists such as thrombin, PAF, TxA₂, ADP, α -adrenergic and β -adrenergic receptors couple G-proteins, Haslam and Davidson (1984*ab*) reviewed in Siess, (1989). Subsequently, activating PLC which in turn hydrolyses PIP₂ to give the two second messengers DAG and InsP₃, (Haslam and Davidson, 1984*ab*; Marks *et al.*, 1990). In permeabilised platelets InsP₃ has been shown to cause secretion, aggregation and protein phosphorylation (Lapetina *et al.*, 1984; Brass *et al.*, 1987) and these events appear to be a consequence of the ability of InsP₃ to mobilise Ca²⁺ sequestered within the platelet dense tubular system (Authi and Crawford, 1985; Nishizuka, 1986; Uemura *et al.*, 1990). As all platelet agonists act through PI, modulation of the PI signalling pathway in platelets could represent an effective way to modulate platelet activity.

1.3 Platelets in health and disease.

The number of circulating platelets in normal persons is maintained within a narrow range and the factors that regulate this count are poorly understood.

However 60- 70% of all platelets are in the peripheral circulation and in dynamic equilibrium with a pool of platelets in the spleen. Extreme alterations in platelet number, too few as in thrombocytopenia, or too many as in thrombocytosis can be detrimental. High platelet numbers as in thrombocytosis are associated with thrombosis or patients may undergo spontaneous aggregation in the circulation; bleeding time may also be prolonged, especially if additional risk factors such as pre-existing arterial disease or prolonged immobility are present. On the other hand low platelet count, a side effect of aggressive chemotherapy or irradiation therapy, causes an inability for blood to clot and there is a danger of death due to excessive bleeding.

Platelets play a crucial role in haemostasis and in the pathogenesis of thrombosis. Their role in haemostasis is two fold: first, the formation of the haemostatic plug at the site of vascular injury and secondly the secretion of vasoactive substances leading to blood vessel contraction and recruitment of other platelets and substances from their granules which are involved in coagulation.

Diseases such as atherosclerosis rarely lead to occlusion, however the presence of a thrombus and the severe atherosclerotic narrowing causes occlusion of the vessel. 90% or more of acute transmural myocardial infarcts are associated with one or more segments occluded by a thrombus. This is caused by the loss or denudation of an area that is normally a thromboresistant endothelial layer and the subsequent exposure of this area to circulating platelets causing adhesion, aggregation, degranulation and the activation of the circulating platelets. This occlusion leads tissue starvation of oxygen and subsequent death in severe cases such as stroke.

Stress appears to be the most common cause of occlusion in vascular disease. During stress, circulating levels of noradrenaline and adrenaline are elevated

and thus potentiate the risk of thrombosis. In order to reduce the long term risks of vascular disease, it is best to address the factors that are associated with the increased risk and not to use anti-thrombotic drugs when patients suffer from angina or have a myocardial infarction.

Hypertension, elevated blood lipids, the presence of other diseases such as diabetes as well as a genetic predisposition and bad living habits all increase the risk of thrombotic disorders.

There has been active research over the last 20 years in the search for compounds that depress platelet reactivity and function, and it is possible to divide such agents into three classes:

I. Receptor antagonists which antagonise the platelet response to specific stimuli by blocking the interaction of the stimulant with its receptor, eg. phentolamine blocks aggregation induced by catecholamines, ATP blocks platelet ADP receptors. However, because of the diversity of agents that can activate platelets and because of the self-amplification process in platelet activation, it is unlikely that any single type of receptor antagonist could prevent platelet thrombus formation when platelets in vivo are exposed simultaneously to several different stimuli.

II. Enzyme inhibitors which are involved in the platelet activation cascade such as aspirin. Aspirin irreversibly acetylates the cyclo-oxygenase enzyme hence blocking it and this enzyme is responsible for the production of prostaglandins and TxA_2 from AA. Thus, inhibiting this enzyme can cause significant inhibition of platelet function if the response depends completely on AA metabolism such as with low doses of collagen (Roth and Majerus, 1975; Patrono, 1989).

III. Stimulators of AC and local anaesthetics; PGI₂, in platelets produced by the vascular endothelium, acts to stimulate AC elevating cAMP levels thus blocking activation. However because cAMP is a ubiquitous intracellular messenger in most cell, the effect of such drugs are not limited to platelets and their potential clinical value as antithrombotic agents is limited. Local anaesthetics are also non-specific, and act by stabilising cell membranes, and at low concentrations can inhibit secretion and aggregation.

However to date, the most significant clinical studies carried out on anti-thrombotic agents is the finding that aspirin has a protective effect against acute myocardial infarction, showing a decreased incidence of death or recurring myocardial infarction (Roth and Majerus, 1975; Smith and Willis, 1971). Aspirin has been convincingly shown to reduce the incidence of vascular occlusion events in a wide range of patients at risk of thrombotic complications (Patrono, 1989), its beneficial effects linked to the suppression of TxA₂-dependent platelet aggregation.

Upon platelet receptor activation, InsP₃ is liberated from the enzymatic cleavage of membrane lipids, and this in turn acts to mobilise intracellular Ca²⁺. Since platelets play a major role in many diseases such as thrombosis, the development of drugs that can block platelet agonists will no doubt be beneficial. However, so many agonists act together to elicit the platelet response and specific antagonists to each agent is not a practical solution. Since all platelet agonists possess the same signalling mechanisms, it is more reasonable to block platelet activation through blocking signalling. Thus, blocking the InsP₃ receptor would be a good example. If such a compound existed, then it is envisaged that it may have a beneficial effect in the above diseases. Platelets were thus used in this study.

given that occlusive vascular disease is the major cause of death in the western world and the knowledge that platelets play a crucial role in this disease.

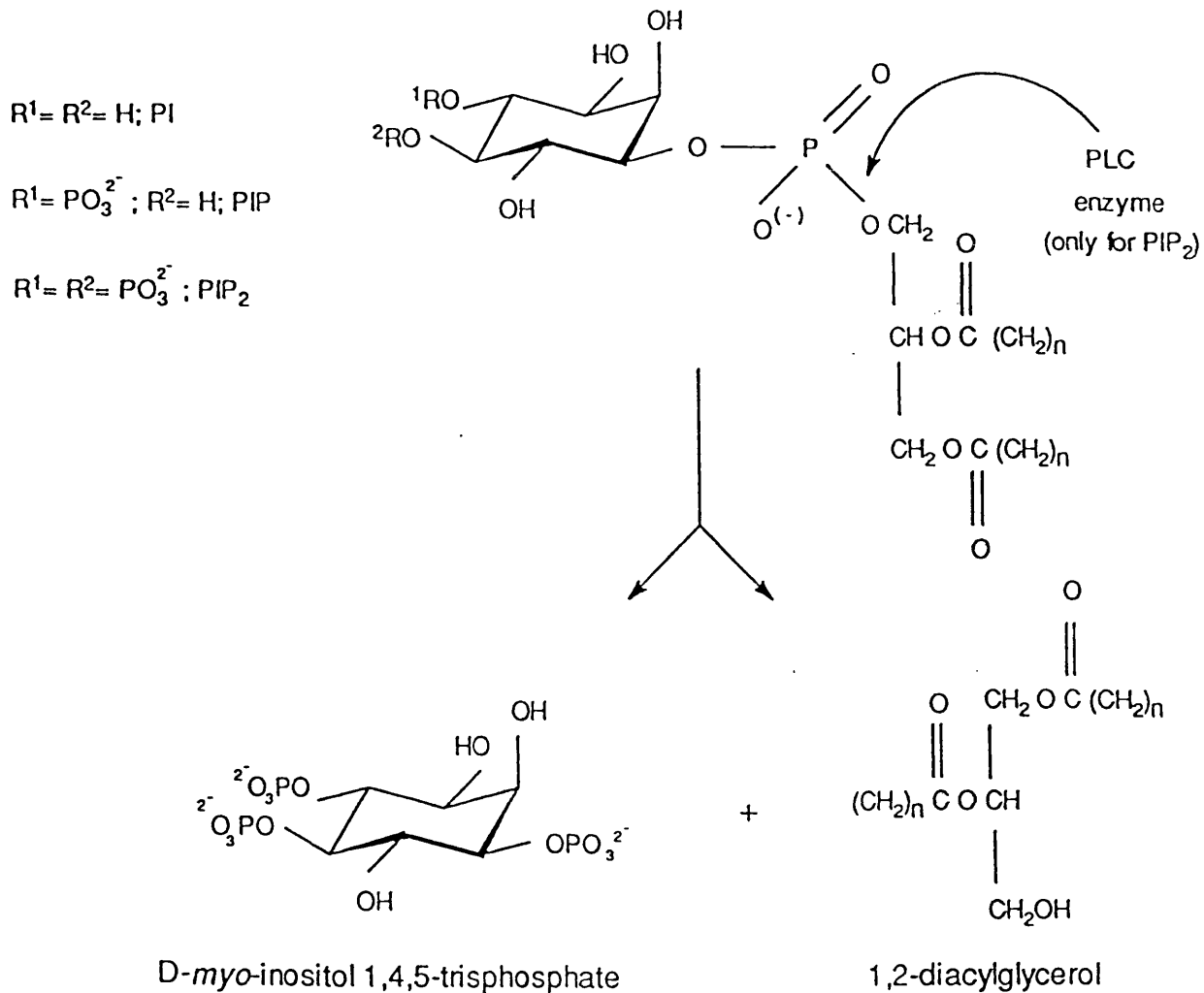


Figure 3. The enzymatic hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) by PLC to give the second messenger molecules $InsP_3$ and DAG.

1.4 A historical aspect of phosphoinositide signalling.

Since the initial findings of Mabel and Lowel Hokin on the effects of neurotransmitters and hormones on phospholipid turnover more than three decades ago, studies on phosphatidylinositol (PI) metabolism have been a focus of attention, Michell *et al.*, (1981), for review see Abdel-Latif, 1986. Thus, it was established that the polyphosphoinositide (PPI) phosphatidylinositol-4,5-bisphosphate (PIP₂) was the one, figure 3.

1.5 DAG, InsP₃ and Ins(1,3,4,5)P₄ as second messengers.

The initial suggestion that stimulated PIP₂ breakdown is involved in Ca²⁺ mobilisation (Berridge and Irvine, 1984; Berridge and Irvine, 1989) has since been confirmed in many cells and tissues. InsP₃ induced Ca²⁺ mobilisation first reported in permeabilised pancreatic acinar cells (Streb *et al.*, 1983) and successive studies confirmed that InsP₃ had similar effects in many cell types (Berridge and Irvine, 1984; Irvine *et al.*, 1986; Berridge and Irvine, 1989).

Analysis of the rates of receptor stimulated InsP₃ formation (Berridge, 1983; Downes and Wusteman, 1983; Tashjian *et al.*, 1987; Horstman *et al.*, 1988) and its subsequent complex metabolism (Berridge *et al.*, 1988; Shears, 1989) further substantiate the role of InsP₃ as an intracellular messenger by demonstrating that it is both formed and inactivated on a time scale commensurate with a role in mediating receptor regulated Ca²⁺ signals (Merritt and Rink, 1987). The response of most cells to Ca²⁺ mobilising stimuli is initially relatively independent of extracellular Ca²⁺, but the sustained elevation of [Ca²⁺]_i is absolutely dependent on extracellular Ca²⁺.

The substantial literature demonstrating that these two phases of response, mobilisation of intracellular Ca^{2+} and stimulated Ca^{2+} entry across the plasma membrane are features of most cells and depend upon receptor stimulated PIP_2 hydrolysis (Putney, 1986; Putney, 1987; Putney, 1990). The link between PIP_2 hydrolysis and stimulated Ca^{2+} entry is not clear although most models ascribe a central role to InsP_3 because there is reasonable agreement that empty InsP_3 -sensitive Ca^{2+} stores are an important control of Ca^{2+} entry either alone (Putney, 1986; Putney, 1990), or in concert with the phosphorylated product $\text{Ins}(1,3,4,5)\text{P}_4$ (Irvine and Moor, 1986; Irvine and Moor, 1987; Morris *et al.*, 1987). This is an important idea since the triggering of Ca^{2+} entry is fundamental to the generation of the Ca^{2+} signals. However, the possible role of $\text{Ins}(1,3,4,5)\text{P}_4$ as a second messenger remains a controversial one, and to date, a specific role for $\text{Ins}(1,3,4,5)\text{P}_4$, whether it acts as a second messenger on its own right, synergises with InsP_3 or mediated Ca^{2+} entry is not clear. The presence of an $\text{Ins}(1,3,4,5)\text{P}_4$ receptor has become apparent and for this reason, $\text{Ins}(1,3,4,5)\text{P}_4$ is perceived to play a significant role in PI signalling.

DAG is a lipophilic molecule, and on release it remains within the plane of the plasma membrane (Nishizuka, 1984; Nishizuka, 1986; Nishizuka, 1988). The amount of DAG accumulated in platelets is considerably higher than the amount of InsP_3 produced. This consideration is important for the proposed two step mechanism of platelet activation (Siess, 1989), where the first step is InsP_3 formation, and Ca^{2+} mobilisation. These events cause platelet shape change, Ca^{2+} mobilisation primes PKC, possibly by placing the enzyme into a strategic position in the plasma membrane. The second step involves the activation of PKC by DAG leading to platelet aggregation and secretion.

1.6 The role of Ca^{2+} in cell activation.

Calcium ions are key second messengers in eukaryotic cells. An absolute requirement for Ca^{2+} during muscle contraction was first observed over 100 years ago when Ringer (1883) observed that a mixture of tap water, rather than distilled water, and sodium chloride was more effective in maintaining cardiac contractility, and he attributed this to the presence of Ca^{2+} ions in tap water.

In 1962, Grette first proposed a role for this ion in stimulus-secretion coupling in platelets (Grette, 1962). In addition, the development of the aggregometer by Born (1962*ab*) and more significantly the of advances made in monitoring intracellular Ca^{2+} by fluorescent molecules such as quin-2 (Tsien *et al.*, 1982) has led to the undisputable role of Ca^{2+} in cellular signalling. Further, a key role for other messenger molecules is often the modulation of Ca^{2+} fluxes where the only compelling documented intracellular role of InsP_3 (a known second messenger molecule) is the mobilisation of this messenger ion (Berridge and Irvine, 1989).

The importance of Ca^{2+} in the transmission of information inside living cells has been a subject of many reviews (Abdel-Latif, 1986; Putney, 1986; Rink and Merrit, 1990). Ca^{2+} is a regulator of many cell functions and its function is most recognised in excitable and secretory cells where functions such as contraction and secretion are controlled by this cation. In the unstimulated cell, the cytosolic Ca^{2+} concentration is $\approx 0.1\mu\text{M}$, which is 10^4 fold lower than the millimolar extracellular Ca^{2+} , and following stimulation, $[\text{Ca}^{2+}]_i$ rises to $1\text{--}10\mu\text{M}$. The low cytosolic $[\text{Ca}^{2+}]$ is maintained at this level by energy dependent Ca^{2+} transport mechanisms that either extrude Ca^{2+} across the plasma membrane or sequester Ca^{2+} within intracellular organelles (Gill and Cheuh,

1985). In most cells, the cytosolic concentration of free Ca^{2+} ions is low $\approx 60\text{-}200\text{nM}$ and therefore only accounts for a small fraction of the total cellular calcium content (Meldolesi *et al.*, 1990).

The mitochondria do not play a role in InsP_3 induced Ca^{2+} -mobilisation (Joseph *et al.*, 1984; Thevenod *et al.*, 1989). They are only significant if intracellular Ca^{2+} rises over $1\mu\text{M}$ at which point mitochondrial Ca^{2+} uptake comes into action in order to limit the extent of $[\text{Ca}^{2+}]_i$ rise (Meldolesi *et al.*, 1990; Rink and Merrit, 1990). The effect of InsP_3 induced Ca^{2+} mobilisation upon receptor activation is not the only one. Evidence suggests that levels of Ca^{2+} remaining within such organelles appear to have important consequences for signalling and regulation within the cells (Putney, 1987; Takemura and Putney, 1989; Putney, 1990).

The ER is thought to play the pivotal role in Ca^{2+} storage. A high affinity Ca^{2+} -ATPase pump is responsible for transmembrane accumulation of high $[\text{Ca}^{2+}]$ which are maintained within the organelle and subsequent Ca^{2+} release occurs through a specific channel upon activation. Specific intracellular Ca^{2+} pump-blocking agents have recently been obtained discussed in (Rink and Merrit, 1990; Capiod *et al.*, 1989), having no effect on plasma membrane ATPases. Thapsigargin (TG) and more recently 2,5-di(tert-butyl)-1,4-benzohydroquinone (tBuBHQ) are two such compounds which inhibit endoplasmic reticulum Ca^{2+} -ATPase. They are proving to be useful tools in the study of intracellular Ca^{2+} release and storage (Rink and Merrit, 1990).

Thus, increases in intracellular Ca^{2+} concentration caused by agonist stimulation trigger a variety of responses in cells from cell differentiation and proliferation, secretion, metabolism and contraction. These increases are key

signals in the platelet response, and the ability to elucidate the role of Ca^{2+} in platelet activation and to modify Ca^{2+} release with the use of inositol phosphate analogues may provide us with useful tools to modify platelet activation. This can be subsequently implemented in disease states such as thrombosis and atherosclerosis.

1.7 InsP₃ and its metabolism.

A second messenger, once released inside the cell, must be efficiently deactivated metabolically in order to terminate its action and return the cell to its basal state in preparation for a new stimulus. Additionally, metabolites of the second messenger may possess other physiological activities within the cell. These aspects have come under close scrutiny in the past few years and it has become apparent that the metabolism of InsP₃ to free inositol, which is then recycled into lipid synthesis, is of considerable complexity generating many inositol phosphates which are often difficult to separate. In addition, this is further complicated by the fact that InsP₃ is not the only hydrophilic product of PLC induced PIP₂ cleavage, PIP₂ cleavage can also occur at the 2-OH of the phosphorus of the inositol ring, giving rise to cyclic inositol phosphate, *D-myo*-inositol-1:2cyclic,4,5- trisphosphate, Ins(1:2cyc,4,5)P₃.

Three major routes of metabolism have been identified, for review see (Berridge, 1987; Shears, 1989), see figures 4 and 5.

1.7.1 Route 1.

The first route involves the action of enzyme InsP_3 5-phosphatase, responsible for the dephosphorylation at the 5-position phosphate, this having been first demonstrated in erythrocytes (Downes *et al.*, 1982). The other enzymes involved are the inositol polyphosphate 1-polyphosphatase and inositol monophosphatase, see figure 4 and 5.

The product of the action of InsP_3 5-phosphatase enzyme is *myo*-inositol 1,4-bisphosphate [$\text{Ins}(1,4)\text{P}_2$]. Since this has no known physiological role in the signalling pathway and does not mobilise Ca^{2+} (Berridge, 1987). Thus, the dephosphorylation must serve solely as an inactivating step. Hence, InsP_3 5-phosphatase enzyme plays a vital role in terminating release of Ca^{2+} by InsP_3 . This enzyme is sensitive to divalent cations in that it has an absolute requirement for Mg^{2+} and is inhibited by silver, zinc and cobalt (Downes *et al.*, 1982; Berridge, 1987). However, this enzyme is insensitive to Li^+ (Downes *et al.*, 1982).

This situation is further complicated by the existence of two types of the InsP_3 5-phosphatase enzyme, a soluble type and particulate type and by the presence of more than one type of soluble enzyme in both brain and platelets (Majerus *et al.*, 1988; Shears, 1989). The significance of the presence of enzyme subtypes is still unclear, for review see Shears (1989). The next step in the metabolism involves the enzyme inositol polyphosphate 1-polyphosphatase. This does not attack InsP_3 or *myo*-inositol 1,3,4,5-tetrakisphosphate [$\text{Ins}(1,3,4,5)\text{P}_4$], but is specific for $\text{Ins}(1,4)\text{P}_2$ and *myo*-inositol 1,3,4-trisphosphate [$\text{Ins}(1,3,4)\text{P}_3$] (see later) (Inhorn and Majerus, 1987; Inhorn and Majerus, 1988). This enzyme is Mg^{2+} dependent and Li^+ sensitive and hydrolyses $\text{Ins}(1,4)\text{P}_2$

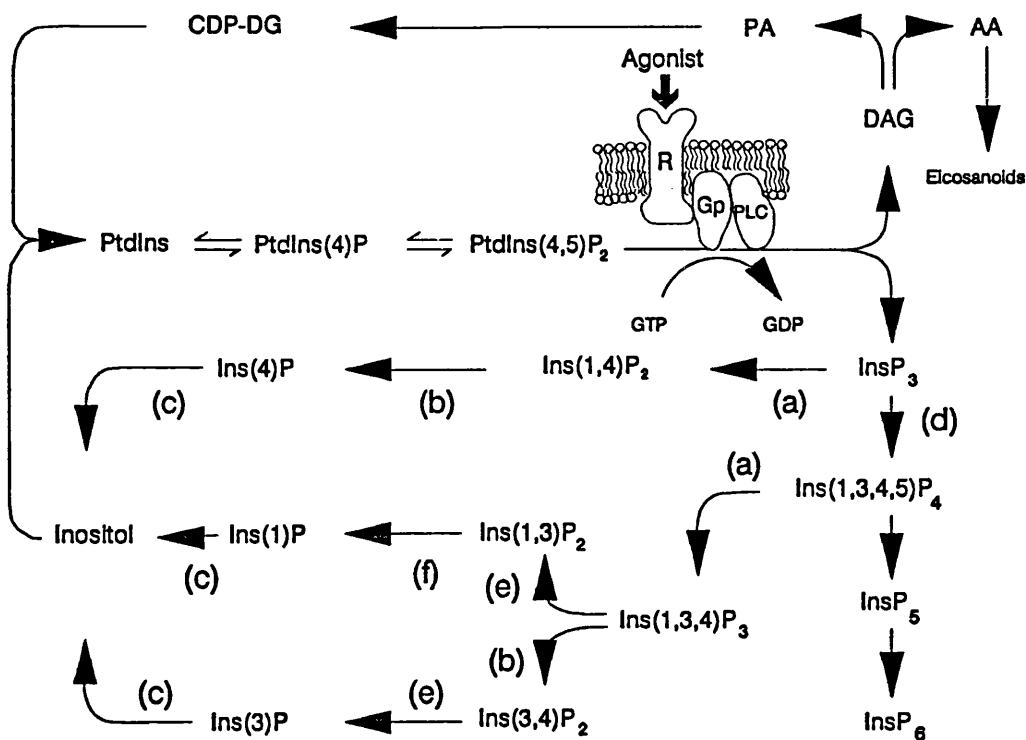


Figure 4. The bifurcating signal pathway involving PIP₂ breakdown to give InsP₃ and DAG. Upon cell surface receptor occupation, a G-protein is activated (G_q) which binds GTP and in turn activates PLC. Both of the messengers produced are metabolized rapidly in order to return the cell to its resting state. a: InsP₃ 5-phosphatase, b: 1-phosphatase, c: inositol monophosphatase, d: InsP₃ 3-kinase, e: 4-phosphatase, f: 3-phosphatase.

to give *myo*-inositol 4-phosphate [Ins(4)P]. Ca^{2+} can also inhibit this enzyme with 40% inhibition occurring at $1\mu\text{M}$ Ca^{2+} (Inhorn and Majerus, 1988).

There have been two reports that Ins(1,4)P₂ can be dephosphorylated to *myo*-inositol 1-phosphate [Ins(1)P] via an inositol polyphosphate 4-phosphatase (Storey *et al.*, 1984; Takimoto *et al.*, 1987), however, Inhorn and Majerus (Inhorn and Majerus, 1987; Inhorn and Majerus, 1988) have proposed that the product of the enzyme reaction was incorrectly identified as Ins(1)P, and it was in fact Ins(4)P. Thus, the major enzyme of metabolism is the 1-phosphatase (Inhorn and Majerus, 1987; Inhorn and Majerus, 1988). Ins(4)P is then dephosphorylated by a nonspecific phosphatase, inositol monophosphatase, which has no specificity for Ins(4)P but also acts on Ins(1)P, *myo*-inositol 3-phosphate [Ins(3)P] and *myo*-inositol 6-phosphate [Ins(6)P]. This enzyme is inhibited by Li^+ (Nahorski, 1990; Kennedy *et al.*, 1990; Kendall and Whitworth, 1990), and the product of hydrolysis is free *myo*-inositol, which can enter the lipid cycle.

1.7.2 Route 2.

Metabolism begins with the phosphorylation of InsP₃ by InsP₃ 3-kinase (Irvine *et al.*, 1988; Irvine *et al.*, 1985; Berridge and Irvine, 1989) to give Ins(1,3,4,5)P₄, and the physiological significance of this molecule has eluded most investigators. The other enzymes involved are the Ins(1,3,4,5)P₄ 5-phosphatase, the inositol polyphosphate 1-phosphatase, the inositol polyphosphate 4-phosphatase, the inositol polyphosphate 3-phosphatase and inositol monophosphatase, see figure 4. The 3-kinase enzyme is mostly soluble in all tissues studied, for review see (Shears, 1989), and has an absolute requirement for Mg^{2+} in order to aid phosphate transfer

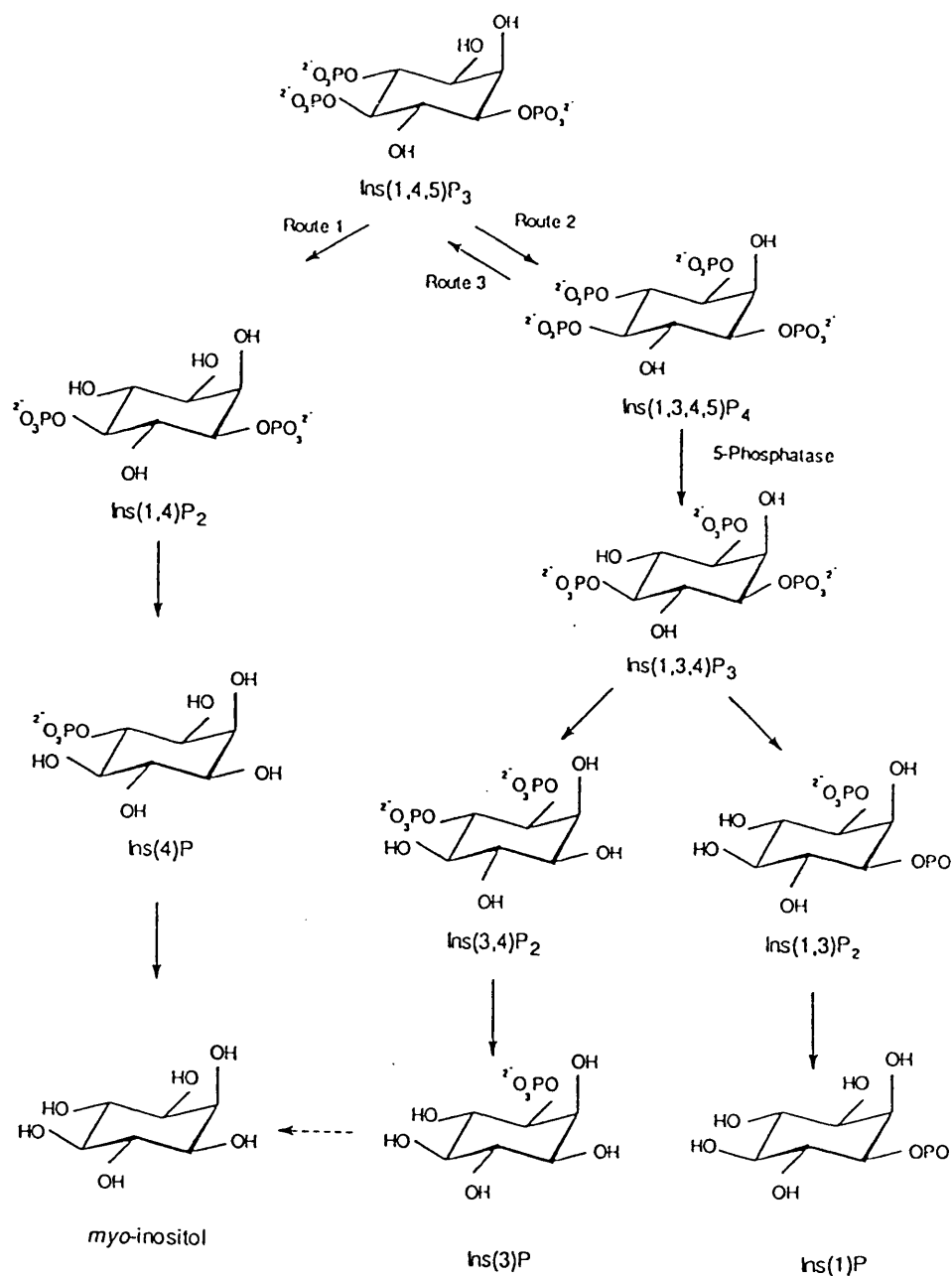


Figure 5. The metabolism of InsP_3 by the two enzymes InsP_3 3-kinase and InsP_3 5-phosphatase and the subsequent breakdown of these metabolites by further enzymes (see text for explanation).

from ATP to the 3-position of InsP_3 . There is evidence that an increase in Ca^{2+} stimulates this enzyme in many tissues in a calmodulin-dependent manner. Thus, transient receptor-mediated elevations in cytosolic Ca^{2+} can increase the rate of $\text{Ins}(1,3,4,5)\text{P}_4$ formation and this may have significant functions (see later).

$\text{Ins}(1,3,4,5)\text{P}_4$ is subsequently metabolized by the $\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase enzyme, which may be similar to InsP_3 5-phosphatase. In brain this enzyme attacks both InsP_3 and $\text{Ins}(1,3,4,5)\text{P}_4$, however, generally different forms will attack InsP_3 or $\text{Ins}(1,3,4,5)\text{P}_4$ preferentially. The soluble type I 5-phosphatase enzyme and the particulate type have higher affinity for InsP_3 over $\text{Ins}(1,3,4,5)\text{P}_4$, thus $\text{Ins}(1,3,4,5)\text{P}_4$ may act as a competitive inhibitor, on the other hand type II soluble enzyme has poor affinity for $\text{Ins}(1,3,4,5)\text{P}_4$. What the significance of this is or how important the inhibition is *in vivo* is difficult to gauge and until these enzymes are fully purified an interrelationship between tissue and enzyme will then become clear.

The product of 5-phosphatase hydrolysis is $\text{Ins}(1,3,4)\text{P}_3$, which can either be phosphorylated by a 6-kinase to give *myo*-inositol 1,3,4,6-tetrakisphosphate [$\text{Ins}(1,3,4,6)\text{P}_4$] (Balla *et al.*, 1987; Shears *et al.*, 1987; Stephens *et al.*, 1990; King *et al.*, 1990) (see later), or dephosphorylated by two enzymes, the inositol polyphosphate 1-phosphatase, a Mg^{2+} dependent enzyme, to give *myo*-inositol 3,4-bisphosphate [$\text{Ins}(3,4)\text{P}_2$] (Inhorn *et al.*, 1987).

The second route is via an inositol polyphosphate 4-phosphatase that gives rise to *myo*-inositol 1,3-bisphosphate [$\text{Ins}(1,3)\text{P}_2$], a Mg^{2+} dependent enzyme that is not inhibited by Li^+ (Bansal *et al.*, 1987; Bansal *et al.*, 1990). Further

metabolism by specific enzymes leads to the monophosphates and subsequently to inositol, see figure 4 and 5.

1.7.3 Route 3.

Recently, reports have indicated the presence of Ins(1,3,4,5)P₄ 3-phosphatase activity (Hoer *et al.*, 1988; Doughney *et al.*, 1988; Cullen *et al.*, 1989) which leads to the reformation of InsP₃ (Shears, 1989). The role of this novel enzyme could therefore be in maintaining InsP₃ levels and the significance of this is not determined. It has been suggested that the substrate for this 3-phosphatase is *myo*-inositol 1,3,4,5,6-pentakisphosphate [Ins(1,3,4,5,6)P₅], in preference to InsP₃. Thus, its actions on InsP₃ only become apparent when this inhibitory effect is removed.

Ins(1,3,4,5)P₄ has also been implicated in the production of Ins(1,3,4,5,6)P₅ by a novel 6-kinase although other evidence has suggested that Ins(1,3,4,5,6)P₅ is produced by the action of a 3-kinase on *myo*-inositol 1,4,5,6-tetrakisphosphate [Ins(1,4,5,6)P₄] (Stephens *et al.*, 1988*ab*) see later section on higher inositol phosphates) (Stephens *et al.*, 1990; Stephens and Downes, 1990).

1.8 Platelet lipids and the PI cycle.

The outer membrane lipid composition of human platelets is phosphatidylcholine (PC) 23%, phosphatidylethanolamine (PE) 11%, phosphatidylserine (PS) 4.5% and PI 5-7%. PPI make up a minor percentage of membrane lipids, phosphatidylinositol 4-phosphate (PIP) and PIP₂ accounting for 15 ≈ 12% of total polyphosphoinositol lipids, reviewed in Siess, (1989) (Mauco *et al.*, 1984; Billah and Lapetina, 1982).

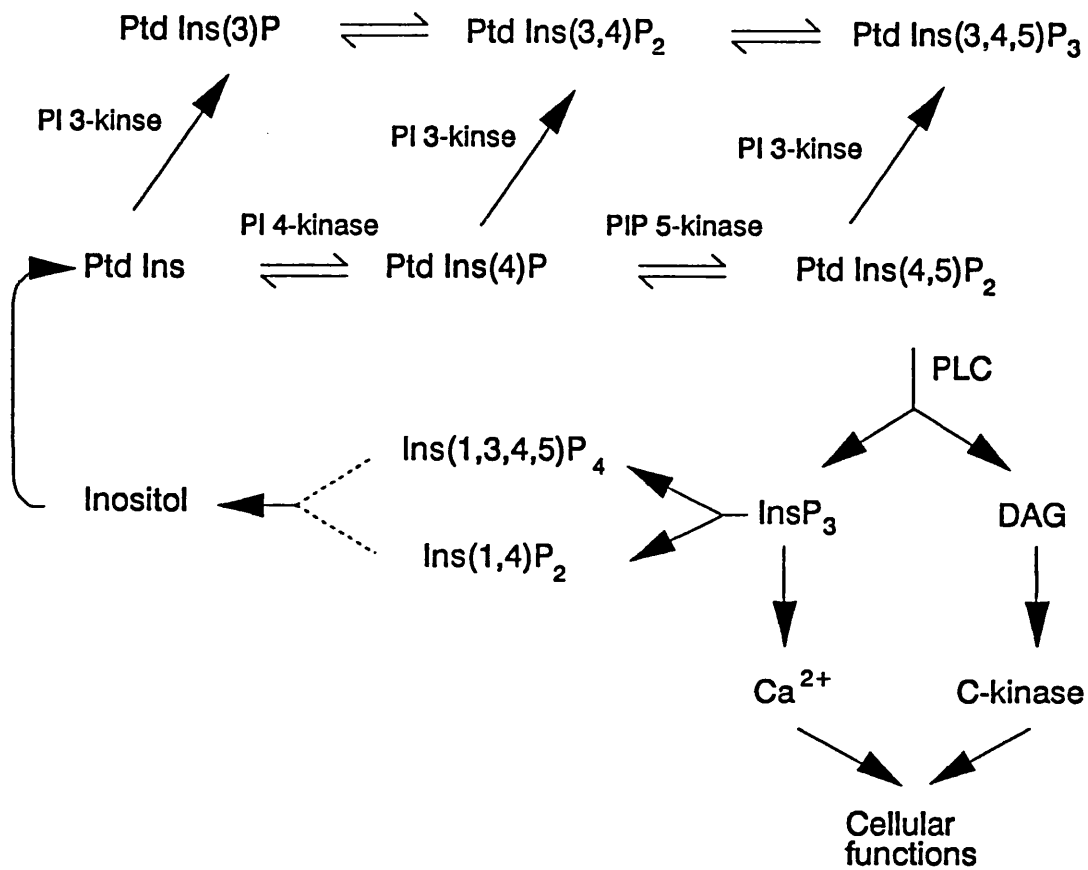


Figure 6. Membrane inositol phospholipids phosphorylation. The classical pathway involving the phosphorylation of the 4-OH and the 5-OH by the two enzymes, PI 4-kinase and PI(4)P 5-kinase, is shown in the lower half of the diagram. Each of these lipids can serve as substrates for the PI 3-kinase giving rise to the novel 3-phosphorylated inositol lipids (shown in the upper half of the diagram).

The PPI's are synthesized by the sequential phosphorylation of PI by the enzymes PI 4-kinase and PI(4)P 5-kinase, both are membrane bound (Kaulen and Gross, 1976). PI and PPI exist in both resting and stimulated platelets in metabolically homogeneous pools. After platelet stimulation with agonists such as thrombin, ADP or AA, a rapid decrease in PIP₂ is observed and after 5 seconds of agonist addition a total of 20- 50% loss of total mass is observed due to PLC activation (Siess, 1989). This loss is transient and 10 seconds after, levels return to normal (Agranoff *et al.*, 1983; Billah and Lapetina, 1982). Levels of PI do not decrease after platelet stimulation, in fact they are found to increase after a lag time of 30 seconds from platelet stimulation (Billah and Lapetina, 1982) due to resynthesis and flux.

Two types of enzymes have been established in the synthesis of inositol lipids, type I kinase [with intrinsic tyrosine kinase activity (Takenawa *et al.*, 1990; Auger *et al.*, 1989)], leading to the phosphorylation at the novel-3 positions, and type II kinase being the classical kinase involved in PIP and PIP₂ production and phosphorylation at the 4-and 5- positions (Sultan *et al.*, 1990; Auger *et al.*, 1989; Cunningham *et al.*, 1990; Takenawa *et al.*, 1990; Whitman *et al.*, 1988), see figure 6.

PI turnover in platelets was first noted by Firkin and Williams, who demonstrated that ³²P-labelled phosphate was incorporated into PA, PI and PPI. In addition, recently, the novel lipid phosphatidylinositol 3-phosphate [PI(3)P] and phosphatidylinositol 3,4-bisphosphate [PI(3,4)P₂] have been shown to accumulate upon thrombin stimulation (Sultan *et al.*, 1990; Cunningham *et al.*, 1990). However a role for these lipids in platelets is still unclear, but a suggested role for these 3-phosphorylated lipids in other cells has been as

second messengers in regulating cell growth and differentiation, for review see Berridge (1990).

1.9 InsP₃ metabolizing enzymes in platelets.

1.9.1 InsP₃ and Ins(1,3,4,5)P₄ 5-Phosphatase.

As in most other cells, platelets metabolize InsP₃ by two enzymes, a dephosphorylation and inactivation by a 5-phosphatase, and phosphorylation through a 3-kinase (Daniel *et al.*, 1987). Two forms of 5-phosphatase enzyme are thought to exist. Initial data suggests that the 5-phosphatase is mainly cytosolic (Connolly *et al.*, 1985), however, recent evidence suggests a particulate enzyme (Vedia *et al.*, 1988). There are multiple isoforms of the inositol polyphosphate-5-phosphatase enzyme distributed between the soluble and particulate fractions and these act on three substrates, InsP₃, Ins(1,3,4,5)P₄ and Ins(1:2cyc,4,5)P₃.

In human platelets, two forms of the cytosolic 5-phosphatase have been isolated (Connolly *et al.*, 1985; Mitchell *et al.*, 1989). Type I enzyme has a molecular weight of 45kDa and requires Mg²⁺. It has greater activity against Ins(1,3,4,5)P₄ as a substrate at low substrate concentration, and consistent with this is that low concentrations of Ins(1,3,4,5)P₄ (1μM) inhibit InsP₃ or Ins(1:2cyc,4,5)P₃ breakdown. This implies that cellular levels of InsP₃ metabolites may be sustained under conditions where Ins(1,3,4,5)P₄ is formed. Type I enzyme is also phosphorylated by PKC (Connolly *et al.*, 1986) and this is associated with an increase in enzyme activity, thus PKC activation tends to reduce levels of InsP₃, terminating signalling.

The type II enzyme has a molecular weight of 75kDa and hydrolyses the same substrates as type I (Mitchell *et al.*, 1989). However, it has a lower affinity for both InsP₃ and Ins(1,3,4,5)P₄ and is not phosphorylated by PKC. It requires Mg²⁺ for activity, and unlike the type I enzyme, metabolites of InsP₃ do not inhibit enzyme activity. Further studies have shown that a membrane bound, particulate enzyme is also present in platelets, and approximately 40% of InsP₃ 5-phosphatase activity is membrane-bound (Vedia *et al.*, 1988), with a molecular weight of 49kDa (Connolly *et al.*, 1985). The existence of 3 types of 5-phosphatase enzyme confounds the simple interpretation of the role of this enzyme in controlling inositol phosphate fluxes and in turn Ca²⁺ signalling. Approximately twice as much type II enzyme is present in platelets as the type I, and since type I enzyme has lower affinity for Ins(1,3,4,5)P₄ than type II, it appears that cellular levels of Ins(1,3,4,5)P₄ may influence which of the phosphatases hydrolyse InsP₃.

Enzyme type	K _m	
	InsP ₃	Ins(1,3,4,5)P ₄
Soluble: Type I	7.5μM	0.5μM
:Type II	24μM	7.5μM
membrane bound	100μM	

Table 1. The two types of 5-phosphatase enzymes in platelets and their affinities for InsP₃ and Ins(1,3,4,5)P₄.

This is further complicated by the fact that type I enzyme is phosphorylated and activated by PKC. Thus type I enzyme hydrolyses InsP₃ > Ins(1,3,4,5)P₄.

Therefore, at low substrate concentration $\text{Ins}(1,3,4,5)\text{P}_4$ is hydrolysed rapidly and competitively inhibits InsP_3 hydrolysis, and at high substrate concentration InsP_3 is metabolized at a greater rate. For a summary see Table 1.

1.9.2 InsP_3 3-Kinase.

The 3-kinase enzyme responsible for the 3-phosphorylation of InsP_3 (InsP_3 3-kinase), is a Ca^{2+} /calmodulin dependent enzyme as for other cell types (Biden *et al.*, 1987; Daniel *et al.*, 1987; Daniel *et al.*, 1988).

In stimulated platelets, $\text{Ins}(1,3,4)\text{P}_3$ is the major metabolite and not $\text{Ins}(1,4)\text{P}_2$ as found in other cells (Daniel *et al.*, 1988), and since this route of metabolism consumes ATP, one can envisage that the metabolites of this pathway may play an important role in platelet function. However, as for as Ca^{2+} mobilisation goes, $\text{Ins}(1,3,4)\text{P}_3$ has been shown to be very poor at releasing Ca^{2+} , if at all (Irvine *et al.*, 1986) and its direct function in platelets or any other cell type has not yet been defined. Further, King *et al.*, (1990) have recently found that thrombin-stimulation of permeabilised platelets causes the accumulation of a novel inositol tetrakisphosphate by the action of a 6-kinase enzyme. The metabolite was identified as $\text{Ins}(1,3,4,6)\text{P}_4$. However, this accumulation is minor in comparison with its precursor $\text{Ins}(1,3,4)\text{P}_3$, and it is thought to play only a minor part (King *et al.*, 1990).

InsP_3 3-kinase is a substrate for PKC (King and Rittenhouse, 1989), and thus receptor occupation will phosphorylate this enzyme increasing its activity, thus removing InsP_3 from the cytosol and its Ca^{2+} mobilising actions. However, Lin *et al.*, (1990) have proposed that PKC inhibits the 3-kinase enzyme, based upon studies using PKC activators and inhibitors.

InsP₃ 3-kinase consists of two polypeptides, 53 and 36 kDa molecular weight, and the majority of activity is associated with the 36kDa component. It is not known if these two different polypeptides are distinct enzymes (Takazawa *et al.*, 1989) or if they share a precursor-product relationship (Lee *et al.*, 1990).

In platelets, Ins(1:2cyc,4,5)P₃ is formed upon thrombin stimulation (Weber and Johnson, 1986; Ishii *et al.*, 1986). This inositol phosphate is capable of mobilising Ca²⁺ (Wilson *et al.*, 1985) in platelets with a similar potency to InsP₃, however little interest in this compound as a second messenger has been maintained due to its low ability to release Ca²⁺ in other cell systems.

1.10 Lithium (Li⁺) as an inhibitor of inositol trisphosphate metabolism.

Lithium is an uncompetitive inhibitor of the 1- phosphatase and inositol monophosphatase (Inhorn and Majerus, 1987; Drummond, 1987; Inhorn and Majerus, 1988), see figure 4. Li⁺ is uniquely effective in the therapy and prophylaxis of manic depression. In the last few years there has been growing evidence that the actions of Li⁺ in affective disorders is related to its ability to interfere with PI signalling (Batty and Nahorski, 1985), for review see (Nahorski *et al.*, 1990). It has been argued that Li⁺ may act by reducing the supply of inositol for the resynthesis of PI's that are used to generate second messengers.

However, until alternative inhibitors of inositol monophosphatase or inositol uptake are shown to be as efficacious as Li⁺ clinically, the therapeutic actions of Li⁺ can not be ascribed to these cellular actions. On a positive note however, the stimulus-dependent effects of Li⁺ on PI signalling at concentrations within

the therapeutic range highlight the potential of targeting drugs at an intracellular second messenger signalling system.

1.11 InsP₃ receptor.

It has been known for some years now that the ability of InsP₃ to open a Ca²⁺ channel in the membrane associated with the endoplasmic reticulum or specialised calciosome depends critically on the positional distribution of the phosphate groups on the inositol ring (Potter, 1990*bc*). It is reasonable to assume therefore, that InsP₃ interacts with a specific receptor linked directly or indirectly to this Ca²⁺ channel.

The initial identification of specific InsP₃ binding proteins was achieved using radioligand binding assays and these utilised membranes from peripheral tissues such as neutrophils, hepatocytes and adrenal gland microsomal membranes (Baukal *et al.*, 1985; Spat *et al.*, 1986), however, due to the low abundance of InsP₃ receptors in these tissues, subsequent studies turned to the CNS where [³H]InsP₃ binding in the cerebellum was detected over 100 times greater than in most peripheral tissue (Worley *et al.*, 1987*ab*; Willcocks *et al.*, 1987; Worely *et al.*, 1989).

The native molecular weight of the InsP₃ binding protein is about 1 million daltons as assessed by gel filtration studies (Supattapone *et al.*, 1988). However, the purified receptor protein is a single 260 kDa (Supattapone *et al.*, 1988) band when analysed on SDS-PAGE, suggesting a tetrameric structure. Upon receptor isolation, most of the biochemical and pharmacological properties of the InsP₃-binding protein remain unaltered, with one exception: [³H]InsP₃ binding to the purified receptor

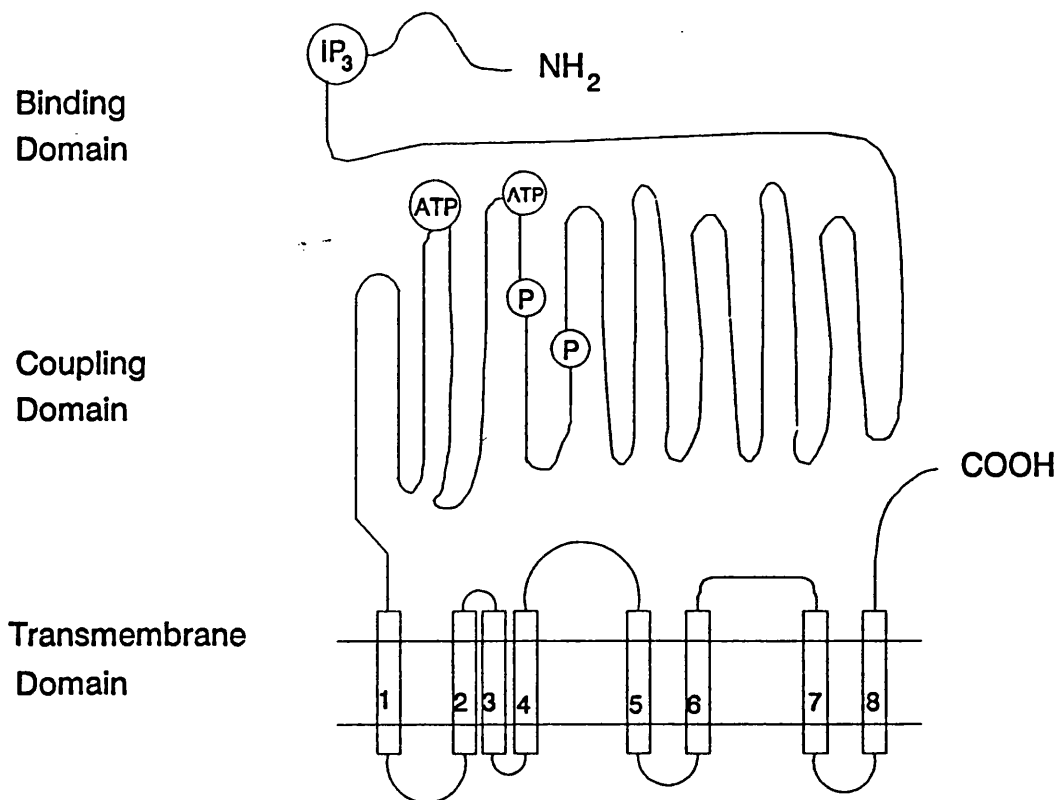


Figure 7. The structure of the InsP₃ receptor. It consists of a large transmembrane protein with eight hydrophobic domains at the carboxy terminus; a large hydrophilic domain that contains the regulatory sites for phosphorylation and the ligand binding domain at the amino terminus (Mignery and Sudhof, 1990).

is unaffected by Ca^{2+} even at concentrations 10000 fold higher than those that inhibit binding in crude membranes (Supattapone *et al.*, 1988), and this property can be reconstituted by the addition of detergent extracted membranes to the purified membrane receptor (Danoff *et al.*, 1988) and the ability of Ca^{2+} to inhibit InsP_3 binding has been proposed to be mediated through a separate protein called "calmedin" (Danoff *et al.*, 1988).

InsP_3 activates Ca^{2+} release, cooperatively, through the direct binding to a ligand activated Ca^{2+} -channel, thus allowing Ca^{2+} mobilisation (Debs *et al.*, 1988; Meyer *et al.*, 1990; Meyer and Stryer, 1990) by the release of Ca^{2+} from intracellular non-mitochondrial pools (Streb *et al.*, 1983). The site of action has been an area of some controversy in recent years and the initial suggestion that the InsP_3 -sensitive Ca^{2+} -pool is part of the endoplasmic reticulum (ER) has recently been questioned, although the bulk of the evidence points to this fact (Streb *et al.*, 1983; Streb *et al.*, 1984; Putney, 1990). The proposal that a novel calsequestrin-containing organelle referred to as "calciosome" is the InsP_3 sensitive- Ca^{2+} pool (Volpe *et al.*, 1988; Meldolesi *et al.*, 1990). However, as yet there does not seem to be any overwhelming evidence for a clearly identifiable and discrete Ca^{2+} storage organelle.

1.12 Regulation of the InsP_3 receptor.

The InsP_3 receptor and the actions of InsP_3 are highly regulated by a variety of agents including Ca^{2+} , pH, PKA and ATP (Worley *et al.*, 1987*ab*; Willcocks *et al.*, 1987; Joseph *et al.*, 1989).

Ca^{2+} was found to be a potent inhibitor of $[^3\text{H}]\text{InsP}_3$ binding with an IC_{50} of 300nM suggesting that Ca^{2+} released by InsP_3 may act as a negative feedback mechanism inhibiting further Ca^{2+}

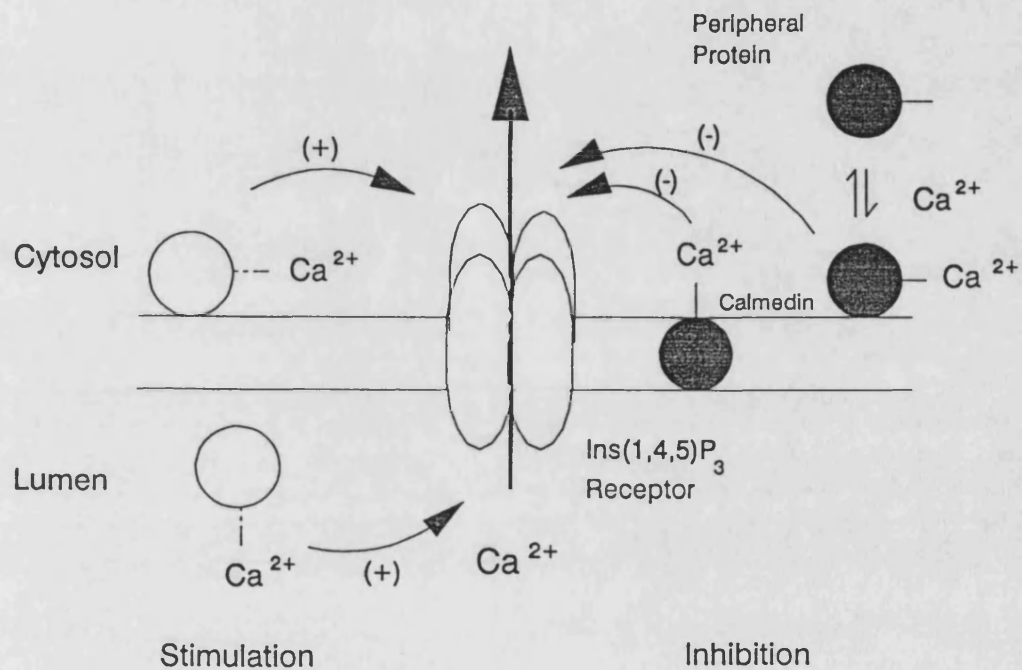


Figure 8. Multiple effects of Ca^{2+} on the InsP_3 receptor. Cytosolic Ca^{2+} can exert both an stimulatory and an inhibitory effect on InsP_3 -induced Ca^{2+} -mobilisation. The inhibitory effects mediated by a membrane calmedin, whereas the stimulatory modes of action are not yet known.

release (Joseph *et al.*, 1989; Zao and Mullan, 1990; Berridge, 1990; Petersen and Wakui, 1990). Calcium has also been shown to have a biphasic regulation on the action of InsP_3 (Iion, 1990), see figure 8.

The receptor can be phosphorylated by protein kinase A (PKA) (Weeks *et al.*, 1984; Walaas *et al.*, 1986). This results in a decrease in the potency in InsP_3 for releasing Ca^{2+} (Supattapone *et al.*, 1988) by phosphorylating the Ca^{2+} -ATPase. Adenine nucleotides can also directly allosterically regulate the InsP_3 -activated Ca^{2+} channels. This allosteric regulation is selective for ATP with other adenine nucleotides being less potent and guanine nucleotides being ineffective (Ferris *et al.*, 1990).

1.13 InsP_3 receptor subtypes and receptor heterogeneity.

InsP_3 binding sites have been described in many tissues. Variations have been reported in the affinity values of these binding proteins to InsP_3 , eg. in the rat cerebellum a reported affinity, $K_d = 40\text{nM}$ (Worley *et al.*, 1987*ab*) compared with $K_d 5\text{nM}$ in bovine adrenal cortex (Baukal *et al.*, 1985). In addition, conflicting reports of InsP_3 binding affinities within a single tissue have come to light, eg. in the liver, Nunn & Taylor (1990) show a K_d of 5.5nM , whereas Guillemette *et al* (1988) show a K_d of 1.7nM and Mauger *et al* find two binding affinities K_d of 2.23 and 88nM (Mauger *et al.*, 1989). Similar discrepancies have been found in other tissues, and Varney *et al.*, (1990) have suggested that although these discrepancies within the affinities of InsP_3 to receptors in different tissues are present, no consistent pattern is present to suggest receptor subtypes.

This confusion regarding the discrepancies within receptor affinities could be simply solved by the discovery of a high affinity receptor antagonist. Heparin, a

sulphated polysaccharide, binds with relatively high affinity, $<2\mu\text{M}$ to the InsP_3 receptor, thus antagonising the actions of InsP_3 and has been used widely as an antagonist (Ghosh *et al.*, 1986; Cullen *et al.*, 1988; Tones *et al.*, 1989). However, it should be noted that heparin has other actions within the cell; it has been found to inhibit the 3-kinase enzyme (Takazawa *et al.*, 1989), to stimulate L-type Ca^{2+} channels (Knaus *et al.*, 1990), to uncouple receptors from their G-proteins including those that stimulate InsP_3 formation (Mousli *et al.*, 1990) and to inhibit InsP_3 3-kinase and $\text{Ins}(1,3,4,5)\text{P}_4$ binding sites. These other effects severely limit the ability to utilise heparin as an InsP_3 receptor antagonist. A second compound, decavanadate, has been found to inhibit InsP_3 -induced Ca^{2+} -mobilisation (Föhr *et al.*, 1989), however this also interacts with $\text{Ins}(1,3,4,5)\text{P}_4$ binding and the enzymes associated with InsP_3 metabolism. Thus to date of starting this study, no specific InsP_3 -receptor antagonists were available.

A recent report indicates that InsP_3 receptors in the liver can exist in two states, a high and low affinity states, regulated by cytosolic Ca^{2+} (Mauger *et al.*, 1989; Pietri *et al.*, 1990). The role of Ca^{2+} is clearest in the cerebellum where increases in $[\text{Ca}^{2+}]_i$ that occur upon stimulation ($0.1\text{--}1\mu\text{M}$) decrease the affinity of the InsP_3 receptor and the sensitivity of Ca^{2+} mobilisation to InsP_3 (Worley *et al.*, 1987*ab*; Joseph *et al.*, 1989). This occurs in the absence of ATP at 4°C thus does not require protein phosphorylation (Supattapone *et al.*, 1988). The same is observed in other tissues, eg. uterine smooth muscle (Varney *et al.*, 1990), neuroblastoma cells (Chueh and Gill, 1986). However, other tissues such as blood platelets (Brass and Joseph, 1985), the adrenal cortex (Varney *et al.*, 1990) and the vas deferens (Mourey *et al.*, 1990) increases in cytosolic $[\text{Ca}^{2+}]_i$ have very little effect on InsP_3 binding to its receptor.

These different sensitivities, to Ca^{2+} , have been explained by the differential distribution of calmedin, a large neutral membrane protein, which confers Ca^{2+}

sensitivity upon purified receptors (Danoff *et al.*, 1988), see earlier). However, the situation is not this simple, because whereas calmodulin is an integral membrane protein, Ca^{2+} sensitivity in a variety of peripheral tissue is irreversibly lost after exposure to low $[\text{Ca}^{2+}]$, suggesting that the Ca^{2+} -binding protein in these tissues may only be loosely associated with the membrane (Zhao and Muallem, 1990).

In platelets, InsP_3 -stimulated Ca^{2+} -mobilisation has been demonstrated (Brass and Joseph, 1985; Adunyah and Dean, 1986) by a ligand binding reaction and InsP_3 -binding proteins have been demonstrated (Hansson *et al.*, 1990). Further little work has been done on this receptor in this tissue regarding cloning and receptor expression. Ligand binding has been shown to increase with an increase in pH, and at high pH two binding sites have been distinguished, one being of low ligand affinity and the second showing high affinity. These data suggest the presence of either more than one receptor subtype or of negative cooperativity. However, binding data alone cannot distinguish between these two possibilities.

Since the InsP_3 receptor has been cloned, a significant homology between itself and the ryanodine receptor found in skeletal muscle has been demonstrated (Fill and Coronado, 1988; Kwan and Putney, 1990; Shah and Part, 1988). The significance of these similarities will only be revealed with future investigations in this area.

1.14 Modified inositol phosphates.

Recognition that D-*myo*-inositol 1,4,5-trisphosphate is a second messenger has stimulated renewed interest in the chemistry of inositol phosphates within the last decade. The major structural requirements for InsP_3 -stimulated Ca^{2+} -

mobilisation are the presence of the 4,5 vicinal phosphates with the additional phosphate at the 1- or 2- positions which increases the affinity (Berridge and Irvine, 1984; Potter, 1990*abc*). *L-myo*-inositol 1,4,5-trisphosphate (*L*-InsP₃), the enantiomer of the naturally occurring molecule, is inactive (Strupish *et al.*, 1988; Taylor *et al.*, 1989). It is important to emphasise that to the date of beginning this study, although inositol phosphates and their analogues available differ widely in their affinities for the InsP₃-receptor and their ability to mobilise Ca²⁺, all the compounds tested appear to be full agonists.

The contributions of the ring hydroxyl groups have been investigated, Henne *et al.* (1988) have studied 1-position modifications, where the phosphate moiety was substituted with bulky ester groups resulting in minimal loss of biological activity. Modifications at the 2-position also cause little or no loss in activity (Hirata *et al.*, 1989; Hirata *et al.*, 1990).

<u>Compound</u>	<u>EC₅₀</u>
InsP ₃	0.13 ± 0.01 μM
6-deoxy-Ins(1,4,5)P ₃	6 μM
<i>scyllo</i> -Ins(2)F(1,4,5)P ₃	0.77 ± 0.08 μM
Ins(2,2)F ₂ (1,4,5)P ₃	0.41 ± 0.05 μM

Table 2. The effect of modifying ring hydroxyls on InsP₃ induced-Ca²⁺ mobilisation. Modification at the 6-OH exemplified by 6-deoxy-Ins(1,4,5)P₃ shows a significant loss in activity as indicated by the EC₅₀, whereas modifications at the 2-OH show little if any loss in Ca²⁺ mobilising activity.

Removal of the 2-OH or its replacement by a fluorine atom show little effect. 2-deoxy-*myo*-inositol-1,4,5-trisphosphate [Ins(2)deoxy(1,4,5)P₃] is 2.5 less potent, DL-2,2-difluoro-2-deoxy-*myo*-inositol 1,4,5-trisphosphate [Ins(2,2)F₂(1,4,5)P₃] is 1.6 fold less potent, and DL-2-deoxy-2-fluoro-*scyllo*-inositol 1,4,5-trisphosphate [*scyllo*-Ins(2)F(1,4,5)P₃] is 3 fold less potent than InsP₃ (Hirata *et al.*, 1989; Safrany *et al.*, 1990), see Table 2.

All these results lead us to assume that the 2-OH cannot be making a major contribution to ligand receptor binding and subsequent activation, otherwise these major alterations in 2-OH functional groups will have had a significant effect on binding and activation.

These data (from Table 3) should be compared with modifications at the 6-OH where D-6-deoxy-*myo*-inositol 1,4,5-trisphosphate [6-deoxy-Ins(1,4,5)P₃] has an EC₅₀ of 6μM (Safrany *et al.*, 1990) and DL-6-methoxy-*myo*-inositol 1,4,5-trisphosphate [6-methoxy-Ins(1,4,5)P₃] has an EC₅₀ of 65μM (Polokoff *et al.*, 1988). Such losses in activity indicate that the 6-OH is important for receptor binding and activation. The 6-OH is a neighbour to both 1- and 5-phosphate groups and removal of this group may effect the conformation of these adjacent phosphates, thus making it weaker, as seen above.

3-Deoxy-*myo*-inositol 1,4,5-trisphosphate [3-deoxy-Ins(1,4,5)P₃] has also been synthesized and it appears to be a full agonist in releasing ⁴⁵Ca from NIH 3T3 cells. Although an EC₅₀ is not yet available, maintenance of activity appears to be probable (Seewald *et al.*, 1990), leading to the perhaps surprising conclusion that the 3-OH is not essential for InsP₃ receptor binding and Ca²⁺ mobilisation. No data are available concerning the conformation of InsP₃ bound to its receptor. While the three phosphates will most likely make the major

contribution to binding energy with the receptor, the three hydroxyls at the 2-, 3- and 6- positions may act as either hydrogen bond donors to the receptor protein or hydrogen bond acceptors from the protein, and from the above modifications it can be seen that both the 6-OH and the 3-OH are important in activity. In addition, multiple deletions of the hydroxyl groups can lead to a substantial loss of Ca^{2+} -mobilising activity as demonstrated by DL-1,2,4-cyclohexane-3,5,6-trisphosphate (loss of ≈ 130 fold activity for the racemic mixture) (Polokoff *et al.*, 1988).

Phosphorothioate analogues, *myo*-inositol 1,4,5-trisphosphorothioate [$\text{Ins}(1,4,5)\text{PS}_3$] and *myo*-inositol 5-phosphorothioate 1,4-bisphosphate [$\text{Ins}(5)\text{PS}(1,4)\text{P}_2$] have both been synthesized. $\text{Ins}(1,4,5)\text{PS}_3$ being metabolically stable and $\text{Ins}(5)\text{PS}(1,4)\text{P}_2$ being still closer to InsP_3 in structure. Both of these analogues are potent mobilizers of Ca^{2+} (Taylor *et al.*, 1988; Taylor *et al.*, 1989) and resistant to the 5-phosphatase enzyme (Willcocks *et al.*, 1988; Csátd and Czarnetzki, 1988; Taylor *et al.*, 1989). However, $\text{Ins}(5)\text{PS}(1,4)\text{P}_2$ is a substrate for the 3-kinase whereas $\text{Ins}(1,4,5)\text{PS}_3$ is not, suggesting that the 4-phosphate may interfere sterically or electronically with the 3-OH, thus inhibiting substrate binding.

$\text{Ins}(1,4,5)\text{PS}_3$ is therefore a persistent activator of the InsP_3 receptor being resistant to metabolic enzymes. As a consequence of this $\text{Ins}(1,4,5)\text{PS}_3$ has been used to demonstrate the synergy between InsP_3 and $\text{Ins}(1,3,4,5)\text{P}_4$ Irvine *et al.* (1988), where in lacrimal acinar cells (Morris *et al.*, 1987) $\text{Ins}(1,3,4,5)\text{P}_4$ was essential in conjunction with InsP_3 in maintaining a Ca^{2+} -dependent K^+ current, dependent upon extracellular Ca^{2+} , $\text{Ins}(1,4,5)\text{PS}_3$ alone gave rise to a single transient response typical of InsP_3 , but together with $\text{Ins}(1,3,4,5)\text{P}_4$ it evoked the same sustained response, confirming that the transient response with InsP_3 was not due to rapid metabolism and that $\text{Ins}(1,3,4,5)\text{P}_4$ was not producing its

effect by protecting InsP_3 against metabolism by the 5-phosphatase (Changya *et al.*, 1989).

Inositol phosphorothioates have found applications in studies of the polyphosphoinositide signalling pathway, discussed in Potter *et al.* (1990*abc*). Thus, chemical modified inositol phosphates and their phosphorothioate analogues are proving to be valuable pharmacological tools in helping to unravel the PI signalling pathway. However the greatest benefit will be gained when a specific small molecule antagonist is identified and to this end many laboratories are working. This will greatly enhance our knowledge of this complex signal transduction pathway, for review see Potter (1990*abc*).

1.15 Permeabilisation methods.

The hydrophilic nature of inositol phosphates and their analogues renders these compounds impermeable to intact cell membranes. Direct access to the intracellular space is however necessary if these compounds are to interact with an intracellular receptor. Thus the cell membrane needs to become permeable and this can be achieved by one of many ways:

1.15.1 Electrical.

When a suspension of cells is exposed to a high electric field, their membranes become permeable to solutes (Knight and Baker, 1982; Baker and Knight, 1978; Knight and Scrutton, 1980). It is thought that the voltage develops across the membrane and causes the membrane to breakdown and become permeable. The magnitude of the potential difference across the cell membrane will therefore depend upon the intensity of the field and the cell radius and will vary with cell size. Thus an electric field applied across cells will cause a breakdown of the

plasma membranes of a homogeneous cell population and will not affect the functional integrity of smaller intracellular organelles. Platelets are very small cells with a diameter of 1- 3 μ m and therefore require a relatively large field strength to permeabilise the cell membrane (Knight *et al.*, 1984; Knight and Scrutton, 1980; Hughes and Crawford, 1990; Knight *et al.*, 1988).

1.15.2 Chemical.

Various chemical agents have been used to provide access into the intracellular compartment of the cell. These include :

α -toxin (Seeger *et al.*, 1984; Thealestam, 1983; McGee *et al.*, 1983); ATP⁴⁻ (Cockcroft and Gomperts, 1979*ab*; Cockcroft and Gomperts, 1980; Gomperts *et al.*, 1983); Detergents. eg. saponin and digitonin (Lapetina *et al.*, 1984).

Saponin was used extensively in this study, and it exerts its action by interacting with the plasma membrane cholesterol, dissolving it, rendering the cells permeable. The use of detergents has been extensive in studying InsP₃-stimulated Ca²⁺-mobilisation in many cell types including platelets (Brass and Joseph, 1985; Bjorck *et al.*, 1986; Diamond and Henrich, 1987).

1.16 Ca²⁺ detection.

Ca²⁺ plays a pivotal role platelet functions. Changes in cytosolic Ca²⁺ concentrations are important for shape change, aggregation and secretory responses, as shown in figure 9.

Free Cytosolic Ca²⁺ is low, being \approx 100nM (Rink *et al.*, 1982) and this is 10000 fold less than the concentration of free Ca²⁺ in the plasma (1mM). Because an increase in

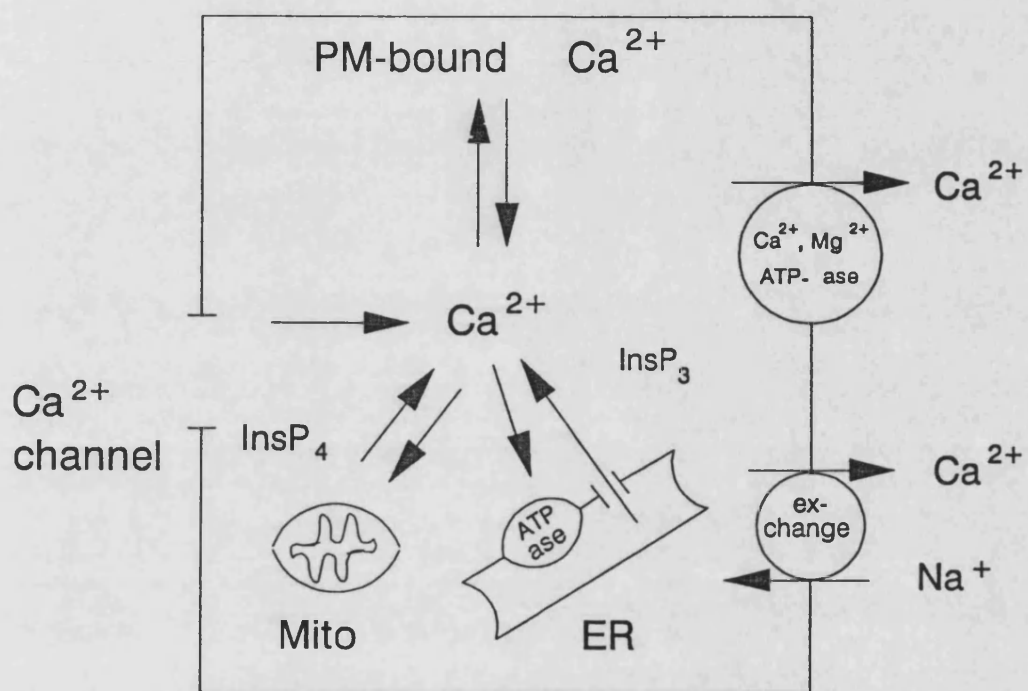


Figure 9. Ca^{2+} pools and Ca^{2+} transport systems in platelets. ER, endoplasmic reticulum; Mito, mitochondrion; PM, plasma membrane.

cytosolic $[Ca^{2+}]_i$ will cause platelet activation, mechanisms in resting platelets exist that limit Ca^{2+} influx from the plasma and actively remove Ca^{2+} from the cytosol. Ca^{2+} released into the cytosol, in stimulated platelets, can originate from either the extracellular plasma via Ca^{2+} channels or from its mobilisation from one or more intracellular pools.

Various methods exist to measure Ca^{2+} fluxes, pools and intracellular concentrations in stimulated and resting cells. The direct measurement of $[Ca^{2+}]_i$ was made possible by the synthesis and subsequent development of Ca^{2+} -fluorescent indicators.

Indicators of free Ca^{2+} ions which can be loaded into cells in a non-disruptive manner initiated a revolution in the studies of intracellular Ca^{2+} homoeostasis. The first of these indicators was quin-2 (Tsien, 1988), the structure of which is based on EGTA, and it selectively binds Ca^{2+} . Subsequently, fura-2 and indo-1 (Grynkiewicz *et al.*, 1985), a second generation of indicators, became available. Quin-2 is not cell permeable, unlike its ester derivative, and the second generation of indicators (Tsien, 1988). Thus the acetomethoxy ester derivatives of quin-2, fura-2 and indo-1 are able to cross cell membranes and once inside the cell, cytosolic esterases cleave the ester groups trapping the indicator in the cell cytosol. Fura-2 is the choice of fluorescent indicator in this study because cell loading requires incubations of 30 minute at 37°C making simple to use in addition to the advantages over quin-2 (see below).

These new indicators have several advantages over quin-2 as indicated by Grynkiewicz *et al.*, (1985):

I. Higher efficiency of fluorescence and increased brightness, reducing the required loading levels thus reducing any buffering of Ca^{2+} transients that is observed with quin-2.

II. Better selectivity for Ca^{2+} ions over other divalent cations.

III. Ca^{2+} binding to fura-2 and indo-1 alters the wavelength of excitation and emission which enables the use of ratio fluorescence at two wavelengths giving more accurate $[\text{Ca}^{2+}]_i$ readings.

IV. Less autofluorescence due to longer wavelength.

V. Lower affinities for Ca^{2+} , which gives a wider window for $[\text{Ca}^{2+}]_i$ measurements.

Tsien and coworkers have since developed a new group of fluorescent indicators based on rhodamine and fluorescein chromophores (Tsien *et al.*, 1982; Minta *et al.*, 1989), fluo-3 is one such dye. It has visible excitation and emission wavelength which are more convenient for fluorescent microscopy and flow cytometry than the UV required by the previous indicators. However, one disadvantage is that upon Ca^{2+} binding it increases in fluorescence without an accompanied increase in wavelength as seen with fura-2 and indo-1, making it unsuitable for ratio fluorescence. The free acids of these dyes are also used to measure $[\text{Ca}^{2+}]_i$, but because they are unable to cross membranes, they need to be used in a permeabilised cell preparation or in a cell homogenate preparation, and fura-2 free acid has been used extensively in this study.

$^{45}\text{Ca}^{2+}$ accumulation in platelets has been studied to identify the organelle responsible for Ca^{2+} sequestration (Brass, 1984). Within the platelet, both the dense tubular system and the mitochondrion can act as Ca^{2+} storage organelles (LePeuch *et al.*, 1983) see earlier, and sequestering is most likely to occur within the dense tubular system (Menashi *et al.*, 1981).

Platelet membrane fractions have demonstrated Ca^{2+} accumulation in the presence of ATP. Subsequently, a Ca^{2+} -dependent ATP-ase was found to be localised in the dense tubular system (Menashi *et al.*, 1981). Hence $^{45}\text{Ca}^{2+}$ is taken up via the Ca^{2+} -dependent ATP-ase pump into the dense tubular system. In addition, further studies show that two distinct Ca^{2+} pumps are associated with the plasma and intracellular human platelet membranes indicating the possible involvement of both Ca^{2+} -ATPases in the regulation of intracellular $[\text{Ca}^{2+}]$ (Enyedi *et al.*, 1986; Enouf *et al.*, 1987). Many studies have been carried out using $^{45}\text{Ca}^{2+}$ as a probe for Ca^{2+} -mobilisation in InsP_3 -stimulated platelets (Brass and Joseph, 1985; Authi and Crawford, 1985) and in the present study, ^{45}Ca measurements are undertaken to assess the mobilisation abilities of inositol phosphate analogues.

The discovery of ion selective membranes for their later use in ion selective electrodes has meant the ability to be able to directly measure Ca^{2+} release in a cell suspension is available. Ion selective electrodes have been widely used to study Ca^{2+} transport and mobilisation in cells (Yamazaki *et al.*, 1979; Streb *et al.*, 1983). The ion selective electrode is based on a liquid membrane of calcium-bis[di(decyl)phosphate] sensor dissolved in dioctylphenylphosphonate and this was an important milestone in Ca^{2+} -ion determination (Craggs *et al.*, 1979). Subsequent modifications and advances within membrane technology has

lead to the design of mini-electrodes and improved sensors based on a neutral carrier system (Ammann *et al.*, 1975; Kopp *et al.*, 1990).

1.17 Functional responses of platelets.

Dense granule release and aggregation are both Ca^{2+} dependent functional responses. Aggregation is dependent on both Ca^{2+} and Mg^{2+} for the binding of fibrinogen to its receptor (see earlier). Thus, aggregation and Ca^{2+} mobilisation should be parallel independent processes. However, to date no studies have been carried out to study the effect of InsP_3 -stimulated aggregation and whether this mimicks Ca^{2+} -mobilisation is not certain. Aggregation is detected using an optical aggregometer (Born, 1962*ab*). A limitation on the use of this aggregometer is that it does not distinguish between primary and secondary aggregation.

Platelets release their granule contents upon stimulation. Granule release can occur either via a weak agonist such as ADP or adrenaline, dependent on the presence of extracellular Ca^{2+} and Mg^{2+} , which causes initial platelet aggregation, AA release and the consecutive formation of TxA_2 and PGs that stimulate secretion, or release can occur independently of extracellular Ca^{2+} with strong agonists such as PAF, thrombin and collagen (Siess, 1989).

In permeabilised platelets, InsP_3 has been shown to induce dense granule release in a dose dependent manner (Brass and Joseph, 1985; Brass *et al.*, 1987), and dense granule release as a measure of platelet function using permeabilised platelets has been assessed in this study.

1.18 AIMS.

Ca²⁺-mobilisation is a common feature of the majority of platelet agonists examined to date. As InsP₃-induced Ca²⁺-mobilisation is the only well characterised system for receptor-operated calcium-mobilisation in platelets, antagonism of the InsP₃ receptor in platelets is a worthwhile therapeutic goal. Therefore the aims of this thesis are:

1. To study Ca²⁺-mobilisation induced by InsP₃ and its analogues in order to develop structure/activity relationships of inositol phosphates.

InsP₃-stimulated Ca²⁺-mobilisation in permeabilised platelets has been studied in many tissues, but little work has been carried out in platelets. The availability of many novel inositol phosphate analogues has made it possible to investigate the Ca²⁺-mobilising abilities of these compounds and thus to establish structure/activity relationships.

2. To identify potential InsP₃ receptor antagonists.

The availability of a specific InsP₃-receptor antagonist would contribute greatly to the understanding of the PI signalling pathway and in this study, we have investigated potential antagonistic activity expressed by the inositol phosphate analogues.

3. To investigate the metabolism of InsP₃ in platelets and the enzymes involved in the metabolism, namely InsP₃ 5-phosphatase and InsP₃ 3-kinase.

InsP₃ is metabolized by a series of complex steps to give active and inactive metabolites. The two main enzymes involved in InsP₃ breakdown are the 5-

phosphatase and the 3-kinase enzymes. In addition, the effect of temperature on InsP_3 metabolism was studied at 4°C, 20°C and 37°C.

4. To determine the effects of inositol phosphate analogous on InsP_3 -metabolising enzymes, namely InsP_3 5-phosphatase and InsP_3 3-kinase.

To examine the effect of metabolic enzyme inhibitors on InsP_3 metabolism. To establish the possibility of the presence of isozymes and to assess the significance of one metabolic route over the other.

5. To establish the functional effects of InsP_3 and analogues on permeabilised platelets in terms of Ca^{2+} release, aggregation and dense granule release.

Elevation of $[\text{Ca}^{2+}]_i$ is known to trigger both aggregation and secretion in platelets. In this study we have attempted to show these effects with the possible aim of elucidating an inhibitor to platelet function.

6. To examine the relationship between the inhibition of Ca^{2+} influx and InsP_3 generation, dense granule release, Ca^{2+} mobilisation and InsP_3 -stimulated Ca^{2+} -mobilisation.

Ca^{2+} influx is thought to play an important role in the Ca^{2+} -response induced by InsP_3 , by refilling the intracellular pools. In this study, the Ca^{2+} -influx inhibitor, SKF 93635, was used to elucidate the role of influx in PAF-stimulated intact platelets, as well as Ca^{2+} mobilisation, dense granule release and InsP_3 production.

MATERIALS AND METHODS

2.1 MATERIALS.

2.1.1 Chemicals.

Bovine serum albumin (BSA) fraction v, protease free; Adenosine 5-trisphosphate (ATP, disodium salt); ethylenediamine-tetraacetic acid (EDTA); ethyleneglycol-bis(b-aminoethylether)N,N,N',N'-tetraacetic acid (EGTA); saponin; heparin, H-3393 and low molecular weight 3000 (H-5271); oligomycin; leupeptin; hepes (acid); mops; quin-2 (free acid) were all purchased from Sigma chemical company, Poole, Dorset, UK.

Calcium chloride (AnalaR); digitonin (AnalaR); D-glucose (AnalaR); magnesium chloride hexahydrate (AnalaR); sodium dihydrogen orthophosphate; sodium hydroxide; potassium hydroxide; sodium succinate (AnalaR); tri-sodium citrate (AnalaR); ammonium sodium orthophosphate; Dow Corning silicone oils 556 and 550 were all purchased from BDH ltd, Avon, UK.

Ionomycin (free acid); Ins(1)P (potassium salt) and Ins(1,3,4,6)P₄ (ammonium salt), were purchased from Calbiochem Company, Novobiochem, UK Ltd. Ins(1,4,5)P₃ (potassium salt), and Ins(1,3,4,5)P₄ (potassium salt) were purchased from The University of Rhode Island Foundation, Kingstone, RI, USA. Nitrocellulose filter discs and GF-B filter strips for the Brandell cell harvester were purchased from Whatman laboratory sales, Maidstone, Kent, UK. Chelex 100 chelating ion exchange resin was obtained from Bio-Rad, U.S.A; α -toxin was obtained from Gibco BRL, Middlesex, UK.

Ammonium hydrogen orthophosphate (AnalR); MeOH (HPLC grade); tetrahydrofuran (THF) (HPLC grade) and diacylglycerol (DAG) were obtained

from Fisons, FSA laboratory supplies, Loughborough, UK. Fura-2 acetomethoxy ester (fura-2 AM) and Fura-2 free acid, calcium calibration kit and 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), were purchased from Molecular Probes, Eugene, Oregon, USA. Platelet activating factor (PAF) was from Bachem Bioscience Inc. Bubendorf, Switzerland. Hypnorm was purchased from Janssen Pharmaceuticals Ltd., Oxon, UK.

SKF 96365 was a gift from Dr C.Poll, Bayer, Slough, UK. All the inositol phosphate analogues tested were provided by Prof. B.V.L.Potter.

2.1.2 Radiochemicals.

D-*myo*-[^3H]-inositol 1,4,5-trisphosphate, [^3H]InsP₃, potassium salt (44 $\mu\text{Ci}/\text{mmol}$; 10 $\mu\text{Ci}/\text{ml}$); Calcium[^{45}Ca] Chloride, ^{45}Ca , (2mCi/ml); [^{14}C]5-hydroxytryptamine creatine sulphate [^{14}C]5-HT, (54mCi/mmol; 50 $\mu\text{Ci}/\text{ml}$) and [^{32}P]gamma-Adenosine 5-trisphosphate, [^{32}P]gamma-ATP, (3Ci/mmol) were obtained from Amersham International, Aylesbury, Bucks, UK.

2.1.3 Buffers.

2.1.3.1 Hepes buffered tyrode (HBT).

		<u>amount</u>
		<u>per litre</u>
NaCl	145mM	8.4g
Hepes	10mM	2.4g
KCl	5mM	375mg
MgCl ₂	1.0mM	200mg
NaH ₂ PO ₄	0.5mM	101mg

The above were dissolved in 950 mls of Milli-Q water and the pH adjusted to 7.4 with NaOH and the volume made up to 1 litre. Prior to use, 5.5mM glucose (100mg/100mls) and 0.25% BSA (250mg/100mls) were added and the mixture stirred gently avoiding any frothing.

2.1.3.2 "Intracellular" like buffer (ICB).

		<u>amount</u>
		<u>per litre</u>
KCl	120mM	89.34g
Hepes	20mM	47.68g
MgCl ₂	6.0mM	12.2g
Sodium succinate	5.0mM	13.52g
KH ₂ PO ₄	2.0mM	2.72g

The above, when dissolved in 1 litre of Milli-Q water, gave a 5x stock which was stored as 50 ml aliquots in plastic containers at -20°C. Prior to use, 10 mls of the stock 5x concentrate was brought to a total volume of 50 mls with Milli-Q water giving the working ICB. 5mM ATP was added and the pH adjusted to 6.9 with 20%w/v KOH (since ATP rapidly decomposes under acidic aqueous solutions, (Dawson and Clarke, 1972) and oligomycin 5 μ l of 5mg/ml stock was also added, and the $[Ca^{2+}]_f$ was adjusted.

2.1.3.3 High K⁺ buffer, (Brass and Joseph, 1985).

		<u>amount</u>
		<u>per litre</u>
KCl	160mM	11.93g
Hepes	13.3mM	3.46g
MgCl ₂	5.3mM	1.08g
ATP	3.3mM	1.67g
Creatine	6.7mM	2.55mg
Phosphate		
Creatine	6.7units/ml	3.53mg
phosphokinase		

This buffer was prepared daily as some of the reagents break down on storage. The above were dissolved in Milli-Q water, the pH was adjusted to 7.1 and the final buffer was stored in a plastic container. This buffer is referred to as buffer A in the text.

2.1.3.4 High K⁺ buffer, (Hughes and Crawford, 1990).

		<u>Amount</u>
		<u>per litre</u>
K ⁺ Glutamate	150mM	27.5g
Pipes	20mM	7.56g
ATP	5mM	2.72g
MgCl ₂	7mM	2.84g
Glucose	5mM	1.00g
EGTA	2mM	20ml of 200mM

This buffer was prepared daily, as required, by dissolving the reagents in Milli-Q water and adjusting the pH to 7.3. This buffer is referred to as buffer B in the text.

2.1.3.5 Platelet membrane buffer.

		<u>Concentration</u>
KCl	30mM	
MgCl ₂	5mM	
Tris-base	20mM	
sucrose	0.3mM	
ATP	1mM	
PMSF	40 μ M	
aprotinin	0.01U/ml	
EGTA	3mM	

These constituents were dissolved in Milli-Q water and the pH was adjusted to 7.0. The buffer was prepared daily as some constituents decompose upon storage. This buffer is referred to as buffer C in the text.

2.1.3.6 Platelet membrane storage buffer.

	<u>Concentration</u>
KCl	0.1M
MgCl ₂	5mM
PMSF	40 μ M
aprotinin	0.01U/ml
EGTA	1mM

The above were dissolved in Milli-Q water and the pH adjusted to 7.2. This buffer was prepared daily to avoid the breakdown of any of the constituents. This buffer is referred to as buffer D in the text.

2.1.4 Drugs and solutions.

1-O-Octadecyl-2-acetyl-sn-glycero-3-phosphocholine (PAF).

A 10⁻²M stock solution was dissolved in chloroform:methanol (4:1, v/v) and stored at -20°C. Sub-aliquots (10 μ l) were removed, dried under nitrogen and made up to 1ml in PBS containing 2.5mg/ml BSA to give a final concentration of 10⁻⁴M PAF. This was stored as 40 μ l aliquoted at -20°C.

Prostacyclin (PGI₂).

PGI₂ was a gift from Dr.B.J.R.Whittle, Wellcome laboratories, Beckenham, Kent. 1mg/ml stock solutions were prepared by dissolving the solid in 0.5M

Tris/HCl buffer (pH 10.5) and 40 μ g/40 μ l aliquots were stored at -20°C. Prior to use, an aliquot was thawed and diluted to 1 ml in 0.01M Tris/HCl buffer (pH 10.5) and stored on ice.

Ionomycin.

A stock solution of 5mM in dry DMSO was prepared and stored at 4°C.

Diacylglycerol (DAG).

1mM stock solution in CHCl₃ was prepared, aliquots of 10 μ l were dried down and stored at -20°C.

Digitonin.

Stock solution of 1mM in 70% ethanol was kept at 4°C.

Ins(1,4,5)P₃ and analogues.

All analogues were provided as the triethylammonium salts freeze dried solids at 0.25- 1.0 μ moles. Stock solutions of 1mM in Milli-Q water were kept at -20°C and subsequent dilutions were carried out with ICB.

Fura-2 AM & Fura-2 free acid.

A 3x 10⁻³M solution of Fura-2 AM was prepared by dissolving in dry DMSO and stored at -20°C. A 1mM Stock solutions of Fura-2 free acid were prepared by dissolving in Milli-Q water and sub-aliquots of 20 μ l were stored at -20°C wrapped in aluminium foil.

Oligomycin.

5mg/ml solutions in ethanol were prepared and stored at -20°C.

All other agonists and reagents were prepared daily as required.

Decavanadate.

The species of vanadate present in aqueous solution is strongly dependent upon the pH of that solution and the concentration of vanadate present. At pH 7.2, vanadate (1.25mmol/l) exists as 50% tetramer, 30% monomer, 10% dimer and 10% other species. At pH 3.75, only decavanadate exists, (Föhr *et al.*, 1989). 0.5M Stock solutions of monovanadate were prepared by dissolving sodium ortho-vanadate (4.6g) in 50ml of 1M KOH. Solutions of KCl (150mmol/l; 2.2g/250ml) and 20mmol/l mops (1.155g/250ml) were prepared in the same flask and the pH adjusted to 7.2. Decavanadate (5mmol/l) was prepared by the addition of 1ml of monovanadate to 99ml of KCl/Mops mixture and the pH titrated to 3.75 giving a distinctive yellow colour of the decavanadate in solution and prior to use the pH was titrated back to 7.2, (Föhr *et al.*, 1989). Decavanadate shows characteristic UV absorbance, and using a UV spectrophotometer it appears as a broad peak between 350 and 400nm.

Ca²⁺sponge.

Commercially available chelating resin (Chelex 100) does not sufficiently lower metal ion contamination from solutions of Ca²⁺ indicator dyes, (Meyer *et al.*, 1990). As a result a heptadentate DTPA-based resin was prepared.

DTPA anhydride (12g, 33.6mmol) was suspended in a mixture of 100ml dry distilled DMSO and 400 ml glacial acetic acid. Dry aminoethyl Bio-Gel P-2 (16g) was then added and the mixture stirred and refluxed for 4 hours at 131°C. The resulting resin was filtered and washed twice with DMSO, twice with 0.1M NaOH, twice with 0.1M HCl and finally with 100 ml Milli-Q water, (Meyer *et al.*, 1990). The resulting resin was left to dry and was then ready for use.

2.2 Methods.

2.2.1 Blood collection.

New Zealand white female rabbits 3.5- 4.5kg were used. Rabbits were sedated with an intramuscular injection of 0.25ml/kg Hypnorm (0.32mg fentanyl citrate/ml and 10mg fluanisone/ml). After 30 minutes, 38ml of blood was collected into plastic tubes, from the ear artery, into 3.2% trisodium citrate (9:1, v/v) using a 19 gauge needle. This was followed by gentle inversion of the tubes.

2.2.2 Preparation of washed platelets.

Washed rabbit platelets were prepared according to the method for human platelets described by Poll *et al* (1986). This method is a modification of the original one described by Blackwell *et al* (1982). The collected blood was centrifuged at 185g for 10 minutes which gave rise to two layers. The platelet rich plasma (PRP) is the top layer and this was retained, discarding the lower red blood cell layer. To the PRP, PGI₂ (300ng/ml) was added and mixed by gentle inversion, the PRP was then centrifuged at 1250g for 15 minutes to give a platelet pellet. After removing the plasma, the pellet was resuspended in HBT and 100ng/ml PGI₂ was added to the suspension. The platelet suspension was centrifuged once again at 1250g for 15 minutes giving a washed platelet pellet.

The platelet suspension, after the final wash was left for 60- 90 minutes to allow the elevated cAMP levels (due to PGI₂ addition) to return to normal and full platelet function to return.

2.2.3 Preparation of permeabilised platelets.

2.2.3.1 Determination of the free $[Ca^{2+}]$ of ICB.

2ml aliquots of ICB were placed in a cuvette, $1\mu M$ fura-2 free acid was added and the calcium concentration of the buffer was determined fluorometrically. Maximal and minimal fluorescence, (F_{max} and F_{min}), were determined and these values used in the equation in the later section 2.2.6. This gives the free Ca^{2+} concentration $[Ca^{2+}]_f$. F_{max} was obtained by the addition of $10mM$ Ca^{2+} , this excess Ca^{2+} binds to the free fura in the cell suspension thus giving a maximal fluorescence. The pH of the suspension was then rendered alkaline with $60-80\mu l$ of $2M$ NaOH and $100-200\mu l$ of $50mM$ EGTA was added to reduce the $[Ca^{2+}]_f$ producing a drop in fluorescence to a minimum. Further additions of $50mM$ EGTA should not produce a further drop in fluorescence. However, if a drop is observed, then sufficient EGTA has not been used and a further aliquote should be added. The changes in fluorescence were recorded and used to give actual Ca^{2+} concentrations in nM (see later section 2.2.6).

EGTA ($40-100\mu l$) of $10mM$ was added to the above working buffer, giving a buffered ICB. A 2ml aliquot of this buffered ICB was placed in a clean cuvette with $1\mu M$ fura-2 free acid and its $[Ca^{2+}]_f$ was determined. If the free concentration exceeded $200nM$, then a further small addition of EGTA ($5-10\mu l$ of $10mM$) was necessary to the working ICB. The aim of this procedure was to reduce the calcium concentration in ICB to mimic intracellular calcium concentrations, thus, concentrations of $100-150nM$ $[Ca^{2+}]_f$ were essential.

Batches of stock ICB were found to require similar amounts of EGTA to reduce $[Ca^{2+}]_f$ to between $100-150nM$ suggesting that the major contaminating source of Ca^{2+} was the ICB's constituent salts.

Whenever ICB is used it refers to a buffer whose Ca^{2+} content has been buffered to between 100- 150nM, unless otherwise stated.

2.2.3.2 Permeabilisation techniques.

2.2.3.2.1 Chemical.

Platelets were washed as described in sections 2.2.2. The suspension was centrifuged at 1250g for 15 minutes and the resulting pellet was resuspended in ICB. The suspension was centrifuged once again and the pellet resuspended to 5mls in ICB and allowed to equilibrate at room temperature for 20- 30 minutes. Saponin 40 $\mu\text{g}/\text{ml}$ (40 μl of 5mg/ml stock) was added and the platelets were mixed for 1 minute and pelleted a final time using the above conditions, and resuspended in ICB to give a corrected final cell count of 10^9 platelets/ml. The permeabilised platelets were then left to recover for 60 minutes prior to use.

When permeabilising using α -toxin, a similar procedure is followed as above. Platelets were washed as described in section 2.2.2, and the suspension was pelleted as above. The pellet was resuspended in buffer A and centrifuged as above. The resulting pellet was resuspended in the same buffer and α -toxin (100 U/ 10^7 cells) was added to the cell suspension and left in contact for 10 minutes. The suspension was then centrifuged and the final pellet resuspended in the high K^+ buffer to a corrected cell count of 10^9 cells/ml, after which the cells were left to recover for 60 minutes. The cells were then ready for use.

2.2.3.2.2 Electrical.

Platelets were washed as described in section 2.2.2, and were pelleted once more. The resulting pellet was resuspended in ICB and centrifuged, at 1250g, as above. The pellet was resuspended in ICB at a concentration of 5×10^9 cells/ml. 500 μ l aliquots were placed in the chamber of the electroporator, already containing a marker such as 1 μ M lucifer yellow or 500cpm [3 H]InsP₃, and exposed to a number of cycles of high voltage discharge (5- 15 cycles at 2- 5kV), after permeabilisation all the aliquots were pooled and the cell count corrected to 10^9 platelets/ml. The cells were then ready for use.

2.2.4 Resealing of electroporated platelets.

Platelets were washed as in section 2.2.2, electroporated as above and the aliquots combined. The platelet suspension was then removed and kept at 37°C. At predetermined time points, 0, 30 and 60 minutes, aliquots of 500 μ l were removed to test for resealing and incorporation of lucifer yellow or [3 H]InsP₃ into the platelets by UV spectrophotometry or liquid scintillation.

2.2.4.1 To test for resealing of electroporated platelets.

Platelets were washed as in section 2.2.2, loaded with [14 C]5-HT, washed a further once in HBT and twice in ICB and resuspended to 5×10^9 cell/ml. 500 μ l aliquots were then porated as above, pooled together and kept at 37°C. At predetermined time points, 0, 30 and 60 minutes, 100 μ l aliquots were removed and challenged with 1mM Ca²⁺ and [14 C]5-HT release was determined (see later section 2.2.7).

2.2.5 Preparation of platelet membranes.

2.2.5.1 Preparation of crude platelet membranes.

Platelets were isolated and washed twice in HBT as in section 2.2.2 and then two times in ICB. Prior to disruption the platelets were centrifuged at 1250g for 15 minutes and resuspended to 10^9 cell/ml in ICB containing $10\mu\text{M}$ leupeptin and $10\mu\text{M}$ pepstatin. The platelet suspension was then rapidly freeze-thawed three times by placing in liquid nitrogen and subsequently at 37°C . The resulting suspension was centrifuged at 25 000g for 60 minutes, and the pellet resuspended in ICB containing $10\mu\text{M}$ leupeptin. The protein concentration of the platelet membrane suspension was determined using the Bio-rad Protein assay (see 2.2.11).

2.2.5.2 Preparation of a pure platelet membrane.

Platelets were isolated and washed as in section 2.2.2, the platelet pellet was suspended in buffer C to a small volume of 5mls. 1ml aliquots were kept on ice and sonicated for 5x 10 seconds. Subsequently, the sonicated aliquotes were pooled together to give the platelet homogenate. The platelet homogenate was centrifuged at 2000g for 30 minutes and the resulting supernatant was kept aside while the pellet was resuspended in buffer C and resonicated as above. The resulting sonicated suspension was centrifuged at 3000g for 30 minutes and the supernatant combined with that of the first sedimentation giving a complete homogenate of platelet membranes. The complete homogenate was then centrifuged at 60 000 for 60 minutes, the resulting pellet was resuspended in 1 ml of buffer C and the protein content determined. The suspension was

subsequently diluted in the storage buffer (buffer D), according to the final protein concentration required, and stored as 500 μ l aliquots at -70°C.

2.2.6 Ca^{2+} measurements in permeabilised platelets.

2.2.6.1 $^{45}\text{Ca}^{2+}$ release assay from permeabilised platelets.

2.2.6.1.1 $^{45}\text{Ca}^{2+}$ uptake and the investigation of permeabilisation.

Washed platelets were prepared as described as in section 2.2.2 and were permeabilised with 20 μ g/ml, 4 μ g/ml and 80 μ g/ml saponin. 15 μ Ci ^{45}Ca (100 μ l of 1 in 10 dilution of stock) was added and uptake into the cells was determined by removing duplicate 100 μ l aliquots, into separate tubes, at timed intervals of 0, 10, 30, 60, 90, 120, 150 and 180 minutes. ^{45}Ca uptake was terminated by the addition of 250 μ l of silicon oil mixture (55%: 45% of fluid 556 and fluid 550), to each tube and the cells were centrifuged through the silicon oil (16 000g, 5 minutes), resulting in the separation of the aqueous and oil phases and giving rise to a pellet which contains the unreleased ^{45}Ca . After centrifugation, the aqueous layer was removed and the tubes inverted, for 20 minutes, to allow the oil to run from the tube, leaving the platelet pellet at the bottom of the tube. 1ml of optiphase scintillant was then added to each tube, and the amount of ^{45}Ca determined by liquid scintillation.

This initial method of centrifuging the platelets through silicon oil proved to be problematic. The platelets were not pelleting in the conventional manner but smearing all over the tube. Subsequently, making it difficult to separate the aqueous layer from the oil layer and not resulting in an intact pellet at the bottom of the tube (possibly due to the adhesive nature of stimulated platelets). The density of the oil was modified to a lower density to allow the easier

movement of cells through the oil, and to a higher density to allow the more uniform movement with a longer time for centrifugation. However, in both cases complete pellet separation proved to be difficult. Thus, an alternative method was employed to achieve separation of the stimulated platelets from their bathing medium. This was achieved using a cell harvester with GF-B filter strips. The platelet suspension was filtered through these strips, and the stimulated platelets with their associated radioactivity would remain on the filter strips. The rest of the unassociated radioactivity going to waste. The amount of ^{45}Ca associated with the stimulated platelets was subsequently determined by liquid scintillation.

2.2.6.1.2 Calculation for InsP_3 - or analogue-induced ^{45}Ca release.

$$\% \text{ } ^{45}\text{Ca} \text{ release induced by } \text{InsP}_3 \text{ or analogue} = \frac{T_b - \text{IP}}{T_b - \text{I}}$$

Where:

T_b is the cpm loaded into permeabilised, untreated platelets ($100\mu\text{l}$; 10^9 cells/ml); IP is the cpm remaining in permeabilised platelets ($100\mu\text{l}$; 10^9 cells/ml) after challenging with InsP_3 or analogue (10nM - $100\mu\text{M}$) for 3 minutes; I is the cpm remaining in permeabilised platelets ($100\mu\text{l}$; 10^9 cells/ml) after ionomycin treatment ($30\mu\text{M}$).

$$\% \text{ Ionomycin-induced } ^{45}\text{Ca} \text{ release} = \frac{T_b - \text{I}}{T_b}$$

Where T_b is the cpm loaded into permeabilised, untreated platelets ($100\mu\text{l}$; 10^9 cells/ml); I is the cpm remaining in permeabilised platelets ($100\mu\text{l}$; 10^9 cells/ml) after ionomycin treatment ($30\mu\text{M}$)

2.2.6.2.3 $^{45}\text{Ca}^{2+}$ release from permeabilised platelets.

Platelets were isolated, washed and permeabilised as in section 2.2.3, resuspended in ICB and loaded with $15\mu\text{Ci } ^{45}\text{Ca}$ and left for 60- 90 minutes (as above). After this time platelets ($100\mu\text{l}$; 10^9 cells/ml) were in contact with $3\mu\text{M}$ InsP_3 , for 0, 5, 15, 30, 45, 60, 180, 300 and 600 seconds, at 4° , 20°C and 37°C and the cell associated ^{45}Ca , for the time course, determined by rapid filtration as above. 100% release was determined by $30\mu\text{M}$ ionomycin and all results were expressed as a percentage of ionophore-releasable ^{45}Ca , see above.

2.2.6.1.4 Optimum cell number.

Washed platelets were prepared as in section 2.2.2, and were resuspended to a cell count of 10^8 , 2×10^8 and 10^9 platelets/ml in ICB. The platelets were then permeabilised with $40\mu\text{g/ml}$ saponin and incubated with $15\mu\text{Ci } ^{45}\text{Ca}$ (see section 2.2.3) for 60- 90 minutes. $100\mu\text{l}$ aliquotes of the permeabilised platelets were then stimulated with $3\mu\text{M}$ InsP_3 for 3 minutes, and the associated radiolabel isolated by rapid filtration. The percentage uptake, and release of ^{45}Ca by the cells by the agonist were determined as above.

2.2.6.1.5 Platelet integrity upon saponin treatment.

Platelets were isolated and washed as in section 2.2.2, loaded with $[^{14}\text{C}]\text{5-HT}$ (see section 2.2.7) and washed a further two times in HBT, twice in ICB and

resuspended to 10^9 cells/ml in ICB, as in section 2.2.2. Platelets were then permeabilised with varying saponin concentrations of 5, 10, 20, 40 and $80\mu\text{g/ml}$ saponin for 1 minute, and [^{14}C]5-HT release, by the permeabilised platelets, was determined as in section 2.2.7.

2.2.6.1.6 Analogue-induced ^{45}Ca -release.

Platelets were washed and permeabilised as in section 2.2.3, resuspended in ICB at 10^9 cells/ml, and loaded with $15\mu\text{Ci}$ ^{45}Ca and left for 60- 90 minutes (as above). After this time platelets ($100\mu\text{l}$; 10^9cells/ml) were challenged with a dose range of the analogue (10nM- $100\mu\text{M}$) for 3 minutes at 4°C or 20°C , to give a dose response curve. The cell associated ^{45}Ca radiolabel was isolated by rapid filtration, and determined by liquid scintillation. An InsP_3 dose response curve (10nM- $30\mu\text{M}$) was also constructed daily in order to assess platelet responsiveness. 100% release was determined by $30\mu\text{M}$ ionomycin and all results were expressed as a percentage of ionophore-releasable ^{45}Ca , see above.

2.2.6.1.7 Testing for antagonistic activity.

Platelets were washed and permeabilised as in section 2.2.3, resuspended in ICB at 10^9cells/ml and loaded with $15\mu\text{Ci}$ ^{45}Ca for 60- 90 minutes (as above). After this time platelets ($100\mu\text{l}$; 10^9 cells/ml) were challenged with mixtures of analogue (10nM- $100\mu\text{M}$) and InsP_3 (300nM at 4°C and $3\mu\text{M}$ at 20°C) for 3 minutes at 4°C or 20°C to give a dose response curve. ^{45}Ca release was terminated after 3 minutes by rapid filtration and ^{45}Ca determined by liquid scintillation. An InsP_3 dose response curve (10nM- $30\mu\text{M}$) was also constructed daily in order to assess platelet responsiveness. 100% release was determined

by 30 μ M ionomycin and all results were expressed as a percentage of ionophore-releasable ^{45}Ca , see above.

2.2.6.2 Fluorometric measurements.

2.2.6.2.1 Platelet preparation.

Platelets were isolated and washed as described in section 2.2.2. The platelets were resuspended in ICB to a corrected cell number of 5×10^9 cell/ml and left to recover for 45 minutes. Platelets were permeabilised with either saponin (40 μ g/ml for 1 minute) or electrically (5- 15 cycles at 5kV) (see section 2.2.3) and resuspended to 10^9 cells/ml. 2mls aliquots of these permeabilised platelets were placed in a quartz cuvette and 1 μ M fura-2 free acid added. The Ca^{2+} signal was calibrated, by obtaining the F_{max} and F_{min} (see section 2.2.3.1), and the basal Ca^{2+} concentration of the permeabilised platelet suspension in the cuvette was buffered to between 100- 200nM. The changes in fluorescence, upon agonist addition, were recorded using a PTi fluorometer (excitation 340 and 380 nM, emission 510nM; 4nm slit width) fitted with a thermostated cuvette compartment and a stirring attachment.

2.2.6.2.2 Calibration of the fluorescence.

This was carried out prior to the experiment by measuring the maximal fluorescence (F_{max}) and the minimum fluorescence (F_{min}). F_{max} was obtained by the addition of 10mM Ca^{2+} . This excess Ca^{2+} binds to the free fura in the cell suspension thus giving a maximal fluorescence. The pH of the suspension was then rendered alkaline with 60- 80 μ l of 2M NaOH and 100- 200 μ l of 50mM EGTA was added to reduce the $[\text{Ca}^{2+}]_{\text{f}}$ producing a drop in fluorescence

to a minimum. Free Ca^{2+} measurements using fluorescent indicators rely on the change in fluorescent properties following Ca^{2+} binding to report the degree of Ca^{2+} saturation of the indicator. At equilibrium, this is related to the free $[\text{Ca}^{2+}]$ according to the mass action equation

$$K_d = \frac{[\text{X}] \times [\text{Ca}^{2+}]}{[\text{CaX}]}$$

which gives;

$$[\text{Ca}^{2+}] = K_d \times \frac{[\text{CaX}]}{[\text{X}]}$$

where;

X is the fluorescent indicator which binds Ca^{2+} ; K_d is the dissociation constant of the fluorescent indicator

Using the fluorescent dye Fura-2 AM, two wavelengths can be used to carry out $[\text{Ca}^{2+}]_i$ measurements and obtain signals which are proportional to Ca^{2+} - bound and Ca^{2+} - free indicator. The ratio of fluorescence at the two wavelengths is directly related to the ratio of the two forms of dye and therefore can be used to calculate $[\text{Ca}^{2+}]_i$. The equation which relates measured Ca^{2+} - bound/ Ca^{2+} -free fluorescence ratio (R) to $[\text{Ca}^{2+}]_i$ is similar to that of the single-wavelength equation;

$$[\text{Ca}^{2+}]_i = K_d \times \frac{R - R_{\min}}{R_{\max} - R} \times \frac{S_{f2}}{S_{b2}}$$

where;

R_{\max} is the fluorescence ratio under saturating Ca^{2+} ; R_{\min} is the fluorescence ratio under free Ca^{2+} conditions; S_{f2}/S_{b2} is the ratio of fluorescence values of

Ca²⁺- bound/ Ca²⁺- free indicator measured at the wavelength used to monitor the Ca²⁺ free concentration.

The main advantage of dual wavelength indicators such as Fura-2 AM is that the absolute concentration of the indicator within the cell is not vital as a ratio of bound/ free indicator is used, thus equivalent loading of cells between days is not essential (Grynkiewicz *et al.*, 1985).

2.2.6.2.3 Modifications on the original technique.

Intact washed platelets were suspended in different buffers prior to permeabilisation. ICB (buffered Ca²⁺ to 100- 150nM and unbuffered), HBT, Buffers A and B were used. In addition, preloading with fura-2 AM, permeabilisation of platelets in the cuvette and electroporation of cells were investigated.

2.2.7 Assessment of permeabilised platelet function, [¹⁴C]5-HT release assay.

5-HT is accumulated into platelet dense granules by several mechanisms, against a concentration gradient (Pletscher, 1986). This phenomenon is utilized in this assay and upon incubating platelets with [¹⁴C]5-HT, accumulation into the dense granule is achieved. Upon activation, dense granules release their contents which includes the radiolabel.

2.2.7.1 Platelet preparation.

Platelets were isolated and washed as described in section 2.2.2. The pellet was subsequently resuspended to a cell number of 8x 10⁸cells/ml and incubated with

200nCi/ml [¹⁴C]5-HT at 37°C for 30 minutes. This was followed by washing the platelets twice in HBT to remove any extracellular [¹⁴C]5-HT and twice in ICB (or high K⁺ buffer A; depending on the experiment). The washed platelets were permeabilised either, electrically (5- 15 cycles at 5kV) or with saponin (40µg/ml for 1 minute) (see results). 500µl aliquots of the permeabilised platelets were transferred to Eppendorf tubes and stimulated with the appropriate agent for 10 minutes at 37°C. Release was terminated by removing 500µl aliquots into ice-cold Eppendorfs containing 40µl of 100mM EGTA. The samples were centrifuged at 12 000g for 5 minutes and two 200µl aliquots of the supernatant were removed into 4 mls of liquid scintillant and the amount of [¹⁴C]5-HT was determined by liquid scintillation.

Percentage release from platelets was then calculated using the following equation (Holmsen & Dangelmaier, 1989):

$$\% \text{ } [^{14}\text{C}]5\text{-HT release} = \frac{\text{Ts}-\text{Bo}}{\text{Tc}-\text{Bo}} \times 100$$

where,

Tc is counts per minute (cpm) in the same volume of untreated, uncentrifuged platelets; Ts is the cpm obtained after centrifugation of stimulated platelets; Bo is the cpm due to the extracellular [¹⁴C]5-HT in untreated, centrifuged platelets.

2.2.7.2 Electroporated cells:

Platelets were isolated and washed in HBT (as in section 2.2.2) and loaded with 200nCi [¹⁴C]5-HT, as above. The washed platelets were then washed twice in HBT, to remove any excess [¹⁴C]5-HT, and twice in ICB, finally resuspended to 10⁹ cells/ml in ICB and left for one hour to recover. 1ml aliquots of the intact platelets were transferred to the perspex chamber of the electroporator and a

current was passed over the cells for 5-10 seconds at varying intensities and number of cycles (5- 15 cycles at 2- 5kV). The treated platelets were then removed from the perspex chamber, and [^{14}C]5-HT release assay was carried out on 100 μl aliquots of the permeabilised cells (as above).

2.2.7.3 Saponin permeabilised cells.

Platelets were isolated washed in HBT (as in section 2.2.2), loaded with 200nCi [^{14}C]5-HT, as above. The platelets were then washed two times in HBT and finally resuspended in HBT to 4×10^9 cells/ml. The platelets were then left for an hour to rest and prior to use diluted 1 in 3 with High K^+ buffer, (Brass and Joseph, 1985). 500 μl aliquots were transferred into eppendorf tubes, treated with saponin, 5 $\mu\text{g}/\text{ml}$ - 80 $\mu\text{g}/\text{ml}$ to ascertain the effect of saponin on platelet 5-HT content (see results). Subsequently, permeabilised platelets were treated with an agonist and the [^{14}C]5-HT release ability of each agonist was evaluated (as above).

2.2.8 Ca^{2+} measurements in platelet membranes.

2.2.8.1 $^{45}\text{Ca}^{2+}$ release assay in platelet membranes.

2.2.8.1.1 $^{45}\text{Ca}^{2+}$ uptake in platelet membranes.

Platelet membranes, crude or pure were prepared as in section 2.2.5 and were used in these experiments. Platelet membranes were loaded with 15 μCi ^{45}Ca and uptake into the membranes cells was determined by removing duplicate 100 μl aliquots at 0, 10, 15, 30, 60, 120 and 180 minutes. The amount of membrane associated ^{45}Ca determined by rapid filtration of the suspension on a Brandell

harvester using GF-B filter strips. The radioactivity was then determined by liquid scintillation and the time-course of ^{45}Ca uptake was determined.

2.2.8.1.2 $^{45}\text{Ca}^{2+}$ release from platelet membranes.

Platelet membranes (crude or pure) were prepared as in section 2.2.5 and loaded with $15\mu\text{Ci } ^{45}\text{Ca}$ for 90- 120 minutes. $100\mu\text{l}$ aliquots of these membranes were incubated with a dose range of InsP_3 (10nM- $30\mu\text{M}$) for 3 minutes and ^{45}Ca release was terminated by rapid filtration as above. 100% release was determined by $30\mu\text{M}$ ionomycin and all InsP_3 induced ^{45}Ca release was expressed as a percentage of ionophore-releasable ^{45}Ca (see section 2.2.6.1).

2.2.8.1.3 The effect of heparin on InsP_3 induced ^{45}Ca release.

Platelet membrane preparations, crude or pure were prepared as above and loaded with $15\mu\text{Ci } ^{45}\text{Ca}$ for 90- 120 minutes. $100\mu\text{l}$ aliquots of these membranes were incubated with a dose range of heparin (100ng/ml- $100\mu\text{g/ml}$) for 3 and 5 minutes, followed by $10\mu\text{M } \text{InsP}_3$ for 3 minutes. ^{45}Ca release was terminated by rapid filtration, and the amount of ^{45}Ca release determined by liquid scintillation, using the equations in section 2.2.6.1.

2.2.8.2 Fluorometric measurements from platelet membranes.

2.2.8.2.1. Calibration of the fluorescence.

The Ca^{2+} content of ICB was determined as in section 2.2.3 and adjusted to 100- 200nM Ca^{2+} . Platelet membranes were prepared as in section 2.2.5. The membrane suspension was then centrifuged at 25 000 rpm for 30 minutes and the protein concentration of the membrane suspension was determined. The

resulting pellet was then resuspended, in ICB, at varying protein concentrations of 0.1mg/ml, 0.3mg/ml and 0.95mg/ml. 2mls of membranes were then transferred to a quartz cuvette and placed in the thermostated compartment of the fluorometer and stirred. 1 μ M fura-2 free acid was then added and the fluorescence signal was calibrated using excess Ca²⁺ (\approx 10mM), to give the maximal signal, and excess EGTA (100- 200 μ l of 50mM) with alkali (60- 80 μ l of 2mM) to give the minimum fluorescence. The changes in fluorescence were recorded on a PTi fluorometer as before and the actual [Ca²⁺] was thus calculated according to the equation of Grynkiewicz *et al* (1985).

2.2.8.2.2 Ca²⁺ measurements in platelet membranes.

Platelet membranes were prepared as in sections 2.2.5. Platelet membranes were centrifuged at 25 000 rpm for 30 minutes and the protein concentration of the membrane suspension was determined. The resulting pellet was then resuspended, in ICB, at a protein concentration of 0.95mg/ml. 2mls of membranes were then transferred to a quartz cuvette and placed in the thermostated compartment of the fluorometer and stirred. 1 μ M fura-2 free acid was then added and the fluorescence signal was calibrated. Agonists were then added to subsequent 2ml aliquotes of membranes in the cuvette, and changes in Ca²⁺ were recorded over time on a PTi fluorometer.

2.2.9 Methods in the study of myo-inositol 1,4,5-trisphosphate metabolism.

2.2.9.1 [³H]InsP₃ metabolism.

Platelets were washed and permeabilised, with 40 μ g/ml saponin, as in section 2.2.3 and were left to recover for 60 minutes. After 60 minutes recovery time, 50nCi [³H]-InsP₃ was added to 100 μ l aliquots of permeabilised platelets and

metabolism was allowed to proceed. Metabolism was terminated after 0, 1, 3 and 10 minutes by the addition of 500 μ l of ice cold chloroform/ methanol (1:1) with added 1% HCl, 200 μ l chloroform, 200 μ l chloroform/ methanol (2:1) and 100 μ l water, (Ward *et al.*, 1992). The samples were then centrifuged at 1400rpm for 5 minutes and the aqueous layer retained. The organic layer was re-extracted with a further 500 μ l chloroform/ methanol (1:1) with 1% HCl, 100 μ l chloroform and 100 μ l water. The sample was centrifuged as above and the aqueous layers combined. The combined aqueous samples were then neutralized to pH 7.5 using 200 μ l 1mM Hepes, 10M KOH (\approx 100- 200 μ l) and 10 μ l universal indicator (to check pH), dried down using a speed vac and finally reconstituted to 200 μ l with Milli-Q water. 100 μ l aliquots were either injected on the HPLC, or stored at -20°C for later analysis on an HPLC.

2.2.9.1.1 HPLC Elution gradient.

Time(min)	A%	B%
0	100	0
5	100	0
45	88	12
60	70	30
61	0	100
65	0	100
66	100	0
90	100	0

A is Milli-Q water; B is phosphate buffer.

Ammonium hydrogen orthophosphate buffer (1.25M) was prepared by dissolving 165 grams in 800 mls Milli-Q water. The pH was then adjusted to

3.8 using orthophosphoric acid and the final volume made to 1 ltr, (Ward *et al.*, 1992).

2.2.9.1.2 The effect of InsP₃ metabolic enzyme inhibitors on [³H]-InsP₃ metabolism.

Platelets were washed and permeabilised, with 40µg/ml saponin, as in section 2.2.3 and left to recover for 60 minutes. 100µl Aliquots of the permeabilised platelets were preincubated with a dose range (0, 3, 10 and 30µM) of the agent [Ins(1,3,5)PS₃, L-Ins(1,4,5)PS₃] or vehicle for 5 minutes, then followed by the addition of 50nCi [³H]-InsP₃. Metabolism was allowed to proceed and terminated after 0, 1, 3 and 10 minutes as above, and the inositol phosphates extracted as above and injected onto an HPLC separation column.

2.2.9.2 Methods to incorporate [³²P]gamma-ATP into *myo*-inositol 1,3,4,5-tetrakisphosphate.

2.2.9.2.1 [³²P]-incorporation into Ins(1,3,4,5)P₄.

Platelets were washed and permeabilised, with 40µg/ml saponin, as in section 2.2.3 and left to recover for 60 minutes. 100µl Aliquots of the permeabilised platelets were then incubated with 1µCi [³²P]gamma-ATP for 1,3,5 and 10 minutes and incorporation was terminated by quenching with 500µl ice cold chloroform/ methanol (1:1) with 1% HCl, 200µl chloroform/ methanol (2:1), 200µl chloroform and 200µl water. The samples were centrifuged at 1400 rpm for 5 min, the aqueous layer retained and the organic layer re-extracted with 500µl chloroform/ methanol (1:1) with 1% HCl (as above). The samples were then centrifuged as above and the aqueous layers combined and neutralised to

pH 7.5 using 10 μ l universal indicator, 1mM Hepes and 10M KOH. The samples were then treated as above.

2.2.9.2.2 The effect of InsP₃ metabolic enzyme inhibitors on [³²P]gamma-ATP incorporation.

Platelets were washed and permeabilised, with 40 μ g/ml saponin as in section 2.2.3 and left to recover for 60 minutes. 100 μ l aliquotes of the permeabilised platelets were pretreated with a dose range (3- 30 μ M) of the analogue [Ins(1,3,5)PS₃ or L-Ins(1,4,5)PS₃ or vehicle] for 5 minutes then 1 μ Ci [³²P]gamma-ATP was added and incorporation of [³²P]gamma-ATP was terminated at 1,3,5 and 10 minutes (as above) and the inositol phosphates extracted, see earlier.

2.2.10 Studies on whole platelets.

2.2.10.1 Fluorometric Ca²⁺ measurements.

2.2.10.1.1 Platelet preparation.

Platelets were isolated and washed and prepared as in section 2.2.2. The platelet pellet was resuspended to a corrected cell count of 8x 10⁸cell/ml and incubated with 2.5 μ M Fura-2 AM for 30 minutes at 37°C. The cell suspension was washed twice in HBT, with the addition of 100nM PGI₂, to remove any excess fura-2. Finally, the platelet count was adjusted to 2x 10⁸ platelets/ml, in HBT, and they were left to recover for 60- 90 minutes.

2ml aliquots of platelet suspension was placed in a thermostatted cuvette (at 37°C) fitted with a stirrer. The Ca²⁺/fura 2 fluorescence was calibrated as

described above (Grynkiewicz *et al.*, 1985). The changes in $[Ca^{2+}]_i$ were recorded using a PTi fluorometer; excitation 340 & 380, emission 510nm, 4nm slit width.

2.2.10.1.2 Calibration of the fluorescence.

Calibration of the Ca^{2+} -Fura fluorescence was carried out as described by Grynkiewicz *et al* (1985). Prior to carrying out an experiment, fluorescence was calibrated by measuring the maximal fluorescence (F_{max}) obtained by lysing platelets with 50 μ M digitonin in the presence of 1mM Ca^{2+} . This causes cell lysis, releasing intracellular fura-2, which comes into contact with excess calcium and binds to it giving a maximal fluorescence. The minimum fluorescence was obtained by rendering the platelet lysate alkali ≥ 8.5 , with \approx 80 μ l of 2M NaOH and chelating the calcium with excess EGTA (100- 200 μ l of 50mM), causing a drop of fluorescence to a minimum. $[Ca^{2+}]_i$ was then determined using the equation in section 2.2.6.2.

2.2.10.2 Dense granule release.

Platelets were isolated and washed as described in section 2.2.3. The platelet pellet was resuspended to 8×10^8 cells/ml and incubated with 200nCi [^{14}C]5-HT for 30 minutes at 37°C. Platelets were washed twice, with the addition of PGI₂, to remove any extracellular 5-HT and the cell count adjusted to 2×10^8 platelets/ml and left to recover for 60- 90 minutes. 500 μ l aliquots of the platelet suspension were transferred into eppendorf tubes and stimulated with a dose range (10nM- 30 μ M) of the appropriate agonist and the assay was carried out as in section 2.2.7.

2.2.10.3 Agonist-induced InsP_3 production from intact platelets.

2.2.10.3.1. Sample preparation.

Platelets were isolated and washed as described in section 2.2.3. The platelets were washed a further 2 times in HBT and the final cell count adjusted to 10^9 cell/ml. The platelets were then left to recover for 60- 90 minutes. Aliquots of $500\mu\text{l}$ were placed in aggregometer cuvettes and the platelets were stimulated with 300nM PAF, with continuous stirring at 37°C . Activation was terminated after 5 seconds with the addition of $100\mu\text{l}$ ice cold 20% perchloric acid. Proteins were allowed to sediment on ice for 20 minutes, followed by centrifugation at $2000g$ for 15 minutes at 4°C . The supernatants were retained and titrated to pH 7.5 with 1.5M KOH containing 60mM Hepes buffer and $5\mu\text{l}$ of universal indicator. Any precipitate KClO_4 was removed by sedimentation at $2000g$ for 15 minutes and the supernatant retained for assay.

2.2.10.3.2 Preparation of InsP_3 standards for $[^3\text{H}]\text{InsP}_3$ binding assay.

A series of eight working $[^3\text{H}]\text{InsP}_3$ standards were prepared ranging from $0.19\text{--}25\text{pmol}/100\mu\text{l}$, by a serial dilution of the 25pmol standard, from an Amersham assay kit, into Milli-Q water. In order to minimise loss of phosphates, plasticware was used throughout the assay, also acidic conditions can hydrolyse and scramble phosphorylated inositols thus neutralization was essential prior to the assay.

2.2.10.3.3 [^3H]InsP₃ production.

The samples and standards, from above, were assayed for InsP₃ using the D-*myo*-inositol-1,4,5-trisphosphate assay system from Amersham. Provided in this kit are; binding protein, assay buffer, tracer and standard.

The reagents in table 3 below were added together in eppendorf tubes, ensuring that the binding protein was the last to be added. The tubes were then vortexed and incubated for 15 minutes on ice, followed by centrifugation at 2000g for 10 minutes at 4°C giving a pellet which contained the bound [^3H]InsP₃. 100 μl aliquots of Milli-Q water and 4 mls of scintillant cocktail were added to each tube, and the radioactivity per sample determined by a β -scintillation counter.

	Volume in μl				
	Tc	NSB	Bo	Std	Spl
Buffer	50	50	50	50	50
H ₂ O	100	-	50	-	-
Standard	-	50	-	50	-
Sample	-	-	-	-	50
Tracer	50	50	50	50	50
Binding prot	-	50	50	50	50

Tc is the total counts in cpm; NSB is the nonspecific binding; Bo is the zero standard; STD is the standard; Spl is the sample. The amount of InsP produced per sample was calculated according to the assay kit, and in all calculations the cpm for NSB has been subtracted from all other counts.

Table 3. Showing the reagents required for [^3H]InsP₃ assay.

2.2.11 Protein assay.

The Bio-rad protein assay was used to determine the protein content of platelet preparations. The Bio-rad assay is a dye binding assay based on the differential colour changes of a dye in response to various concentrations of protein, utilising the absorbance maximum of the acidic solution of coomassie brilliant blue G-250 (used in the assay) which shifts from 465nm to 595 nm when binding to a protein occurs, (Bradford, 1976). Thus, this assay was used as the absorbance of the dye-protein complex is more stable it is much easier to carry out, requiring only one reagent and a short incubation time and finally, it is free from most of the interferences which limit the application of the other assays.

2.2.11.1 Sample preparation.

Samples of platelets were washed twice in BSA free HBT and resuspended to the appropriate cell number of 10^8 , 3×10^8 or 10^9 cells/ml. The suspensions were then exposed to a rapid freeze thawing process for maximum cell disruption by immersion in liquid nitrogen and defrosting at 37°C in a water bath. Duplicate $100\mu\text{l}$ aliquots were assayed against a standard curve.

NOTE

The number of times each experiment was performed is given in the respective figure legends, and where possible, the data are given as the mean \pm SE. When error bars are not seen on a figure, they are smaller than the symbol size.

RESULTS

3.1 ^{45}Ca -mobilisation by InsP_3 and analogues to develop structure/activity relationships and to aid identification of an InsP_3 receptor antagonist.

3.1.1 Optimising ^{45}Ca release assay conditions.

3.1.1.1 The effect of saponin on platelet integrity.

In order to investigate the effect of saponin on platelets, platelets were loaded with [^{14}C]5-HT, suspended in ICB buffer having $[\text{Ca}^{2+}]_i$ of 100-200nM and treated with a dose range of saponin, for set time intervals after which time [^{14}C]5-HT release was determined (figure 10).

It was found that the low concentrations of saponin (5, 10 and 20 $\mu\text{g}/\text{ml}$) had an insignificant effect on platelet [^{14}C]5-HT loss over the 10 minute incubation time period (figure 10). 40 $\mu\text{g}/\text{ml}$ saponin was a little more disruptive where incubations of 5 minutes caused 35% of dense granule contents to be released reaching a maximum of 45% release at 10 minutes. Worse still was 80 $\mu\text{g}/\text{ml}$ saponin where 40% release occurred after incubations of 1 minute, which increased with time reaching a maximum of 65% after 10 minute incubations.

A concentration is required that will permeabilise the cells but have no adverse effect on the platelet structure, intracellular stores and platelet function, thus a concentration of 20 $\mu\text{g}/\text{ml}$, 40 $\mu\text{g}/\text{ml}$ and 80 $\mu\text{g}/\text{ml}$ were used for further investigation.

3.1.1.2 Optimal saponin concentration for ^{45}Ca loading.

Platelets were permeabilised with 20 $\mu\text{g}/\text{ml}$, 40 $\mu\text{g}/\text{ml}$ and 80 $\mu\text{g}/\text{ml}$ saponin for 1 minute and ^{45}Ca uptake determined at set time points over a period of 3 hours. With all the saponin concentrations tested, ^{45}Ca loading reached a plateau after 60 minutes and remained at this level for the following 2 hours (figure 11).

However 20 $\mu\text{g}/\text{ml}$ saponin only resulted in a maximal of

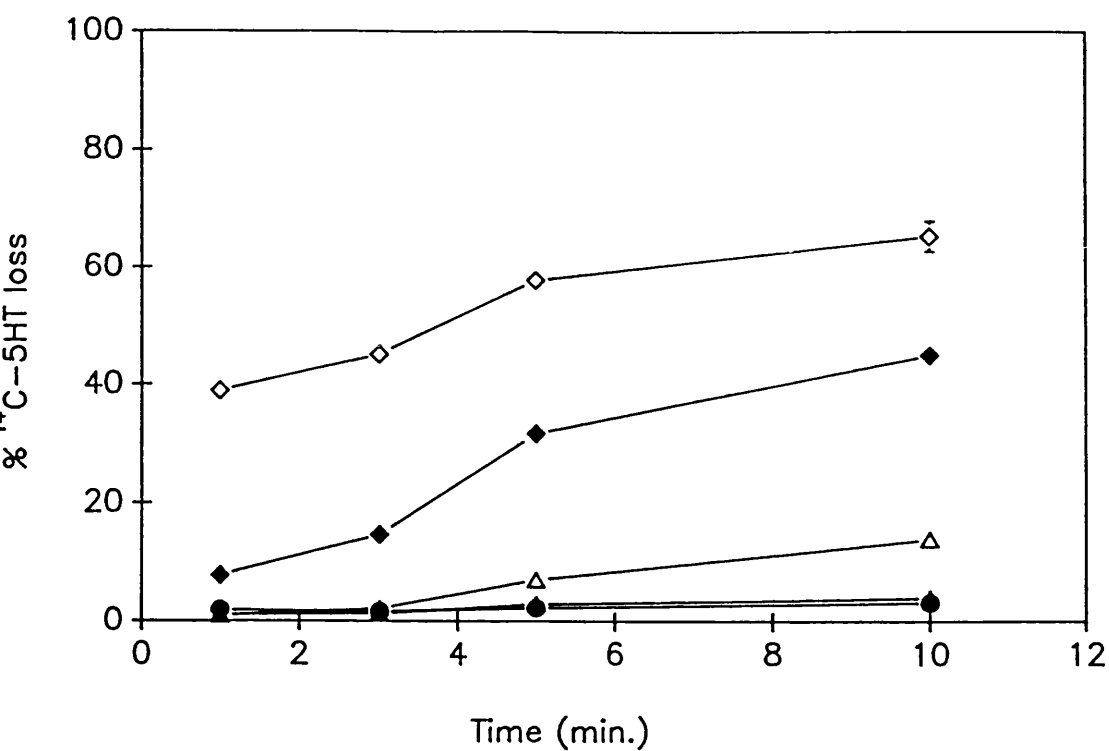


Figure 10. The effect of saponin on dense granule integrity. Platelets were loaded with [^{14}C]5-HT and treated with various saponin concentrations, 5 (●), 10 (▲), 20 (△), 40 (◆) and 80 $\mu\text{g/ml}$ (◇) for 1, 3, 5 and 10 minutes and degranulation was determined. Each point is mean \pm SE of three determinations.

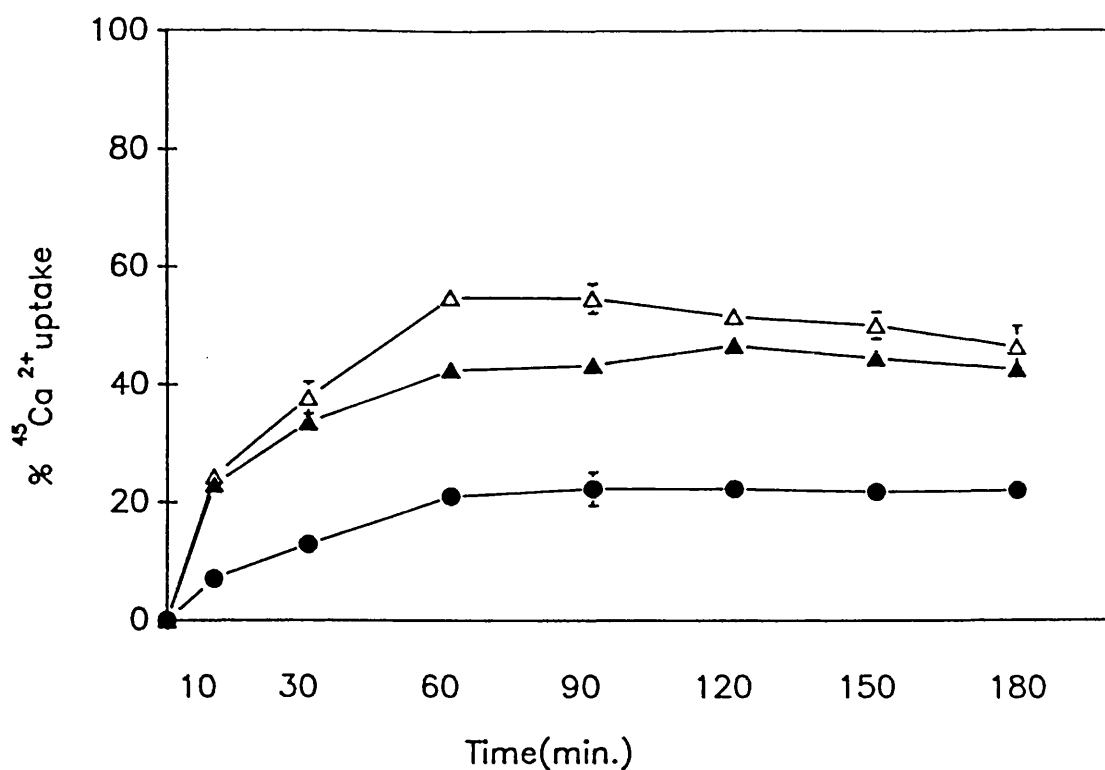


Figure 11. Optimal saponin concentration for ^{45}Ca uptake and time course. Platelets were permeabilised with 20 (●), 40 (▲) and 80 µg/ml (Δ) saponin for 1 minute and ^{45}Ca uptake into permeabilised platelets was determined over a period of 3 hours. Each point is mean \pm SE of three determinations.

20% loading compared with 40% when using 40 μ g/ml saponin and 50% when using 80 μ g/ml saponin. Therefore 20 μ g/ml, saponin although mild on platelets in terms of its integrity, produces low loading. Platelets treated with 80 μ g/ml saponin load only 10% better than those treated with 40 μ g/ml saponin, and when the other effects of saponin are taken into account (see a above for 5-HT data) it was concluded that 40 μ g/ml saponin was the dose of saponin to use.

3.1.1.3 Time course of 3 μ M InsP₃-induced ⁴⁵Ca release.

Platelets loaded with ⁴⁵Ca were incubated with 3 μ M InsP₃ for different times (0-10 minutes) at 4°C, 20°C and 37°C and ⁴⁵Ca release was determined by rapid filtration of the cell suspension. At 37°C, InsP₃ release is maximal after 5 seconds and rapidly decreases to zero at 5 minutes, figure 12, at 20°C, InsP₃ release shows a different profile with maximal release occurring at 45 seconds reaching a plateau of 45% release after 60 seconds which gently decreases to 35% at 10 minutes. At 4°C, a peak of InsP₃-induced ⁴⁵Ca release is reached at 5 seconds, reaching a plateau of 62% release after 60 seconds and slowly decreasing to 57% at 10 minutes. For subsequent experiments, InsP₃-induced release was terminated at 3 minutes by rapid filtration.

3.1.1.4 Optimal cell number for ⁴⁵Ca release assay.

Platelets resuspended to 10⁸, 2x 10⁸ and 10⁹ platelets/ml were permeabilised with 40 μ g/ml saponin for 1 minute, loaded with ⁴⁵Ca for 60 minutes and stimulated with 3 μ M InsP₃ at 20°C for 3 minutes, figure 13. All the platelet preparations loaded ⁴⁵Ca to the same extent (40- 45%), however with 10⁸cells/ml 3 μ M InsP₃ released only 3% of the loaded ⁴⁵Ca, 2x 10⁸cells/ml released 7% but 10⁹cells/ml released 37% of the loaded ⁴⁵Ca. It therefore appears that the greater the concentration of the cells the better the response. Thus ,10⁹ platelets/ml were used in this assay.

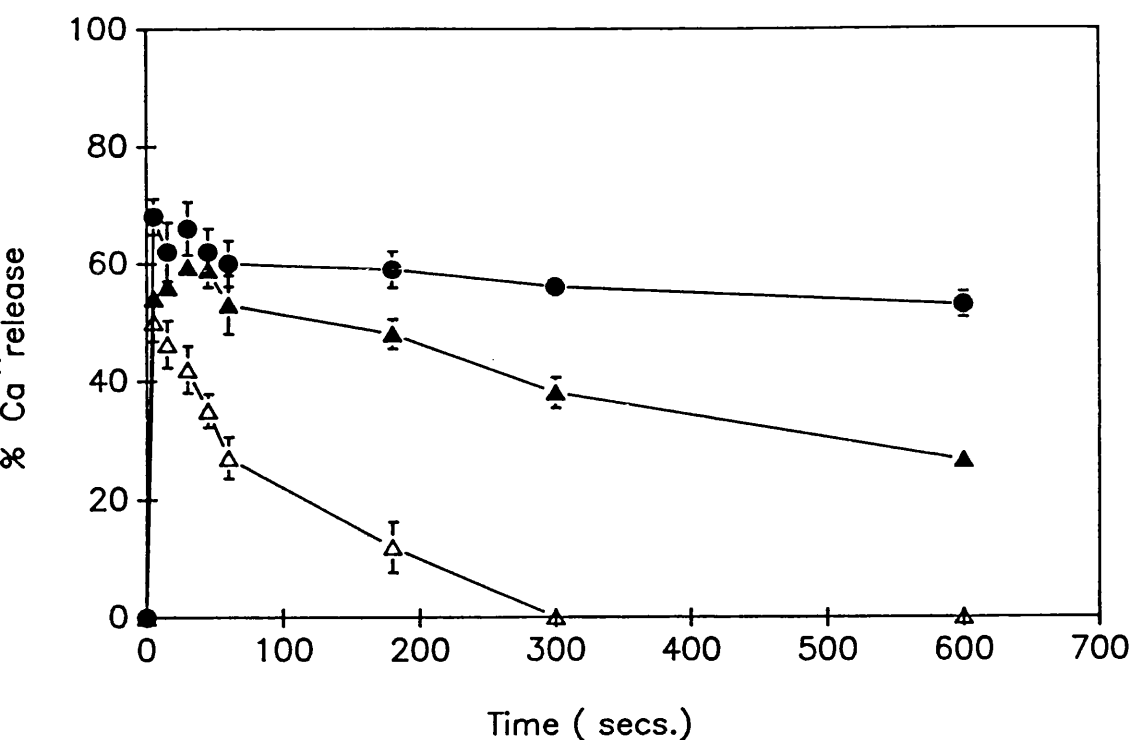


Figure 12. Time course of InsP_3 -induced ^{45}Ca -release at 4°C (\bullet), 20°C (\blacktriangle) and 37°C (Δ). Permeabilised platelets were loaded with ^{45}Ca , stimulated with $3\mu\text{M}$ InsP_3 for 5, 15, 30, 45, 60, 180, 300 and 600 seconds. ^{45}Ca release at each of these time points and at the three temperatures was determined in order to ascertain the optimal contact time between InsP_3 and the permeabilised platelets. Each point is mean \pm SE of three determinations.

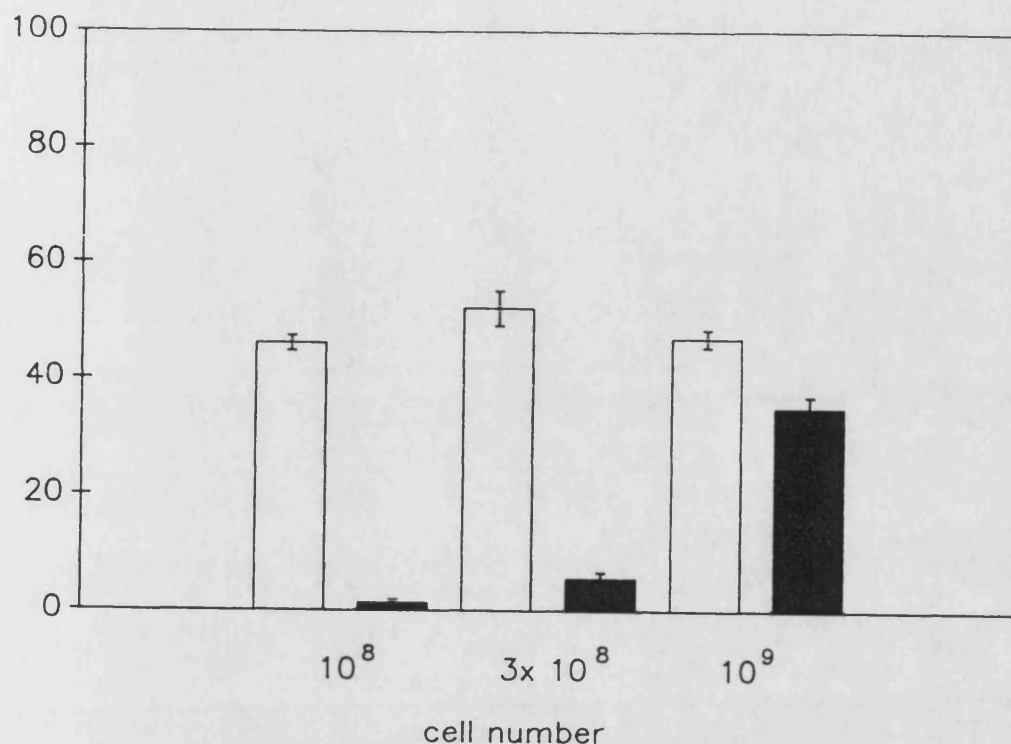


Figure 13. Optimal cell number required for ⁴⁵Ca release assay. Platelets were permeabilised and resuspended to 10⁸, 2x10⁸ and 10⁹ cells/ml, loaded with ⁴⁵Ca and subsequently challenged with 3μM InsP₃ for 3 minutes. ⁴⁵Ca uptake (open bars) and subsequent release by InsP₃ (closed bars) were determined by rapid filtration. Each point is mean ± SE of three determinations.

Permeabilised platelets exhibit ATP-dependent sequestration of ^{45}Ca . Ionomycin ($30\mu\text{M}$) release $90 \pm 3\%$ ($n=10$) of sequestered ^{45}Ca , suggesting that the Ca^{2+} was intravesicular.

3.1.2 Inositol phosphate analogues and ^{45}Ca release.

3.1.2.1 InsP_3 and its enantiomer *L*-*myo*-inositol 1,4,5-trisphosphate (*L*- InsP_3).

At 20°C , InsP_3 shows full activity releasing $80 \pm 4\%$ of loaded ^{45}Ca , however *L*- InsP_3 did not release ^{45}Ca thus having no activity, see figure 14. InsP_3 is the naturally occurring active compound that binds to the InsP_3 -receptor. However, *L*- InsP_3 does not have the ability to bind the receptor or binds only very weakly, thus rendering it unable to release Ca^{2+} , see appendix for structures ($n=6$, mean $\pm\text{SE}$). Thus InsP_3 -induced Ca^{2+} -release from permeabilised platelets was found to be stereospecific.

Compound	EC_{50} at 20°C
InsP_3	$1.14 \pm 0.2\mu\text{M}$
<i>L</i> - InsP_3	$\geq 30\mu\text{M}$

3.1.2.2 6-positional modification.

Myo-inositol-6-deoxy 1,4,5-trisphosphate [6-deoxy- $\text{Ins}(1,4,5)\text{P}_3$], its phosphorothioate analogue, *myo*-inositol-6-deoxy 1,4,5-trisphosphorothioate [6-deoxy- $\text{Ins}(1,4,5)\text{PS}_3$] and its two synthetic precursors, *myo*-inositol-6-

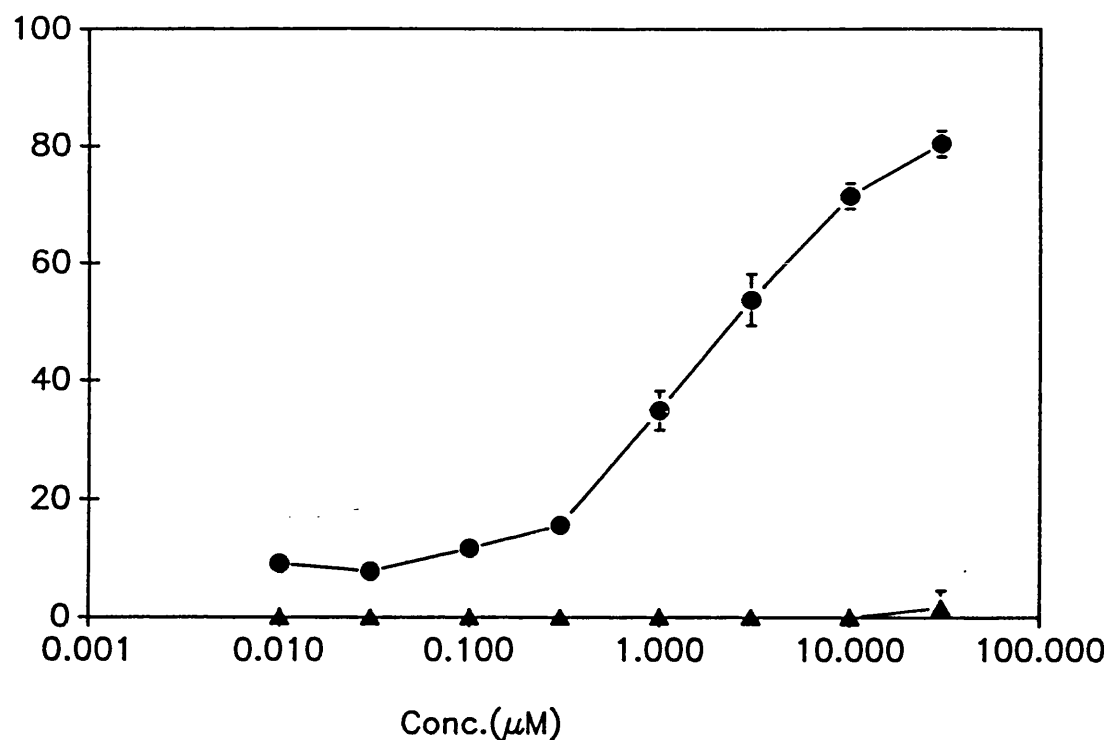


Figure 14. The dose response curves for ^{45}Ca -release from permeabilised rabbit platelets by D-InsP₃ (●) and its enantiomer L-InsP₃ (▲) at 20°C. Saponin permeabilised platelets were loaded with ^{45}Ca in the presence of mitochondrial uptake inhibitors, incubated with InsP₃ or analogue for 3 minutes and the cell associated ^{45}Ca determined by rapid filtration. Each point is mean \pm SE of three determinations.

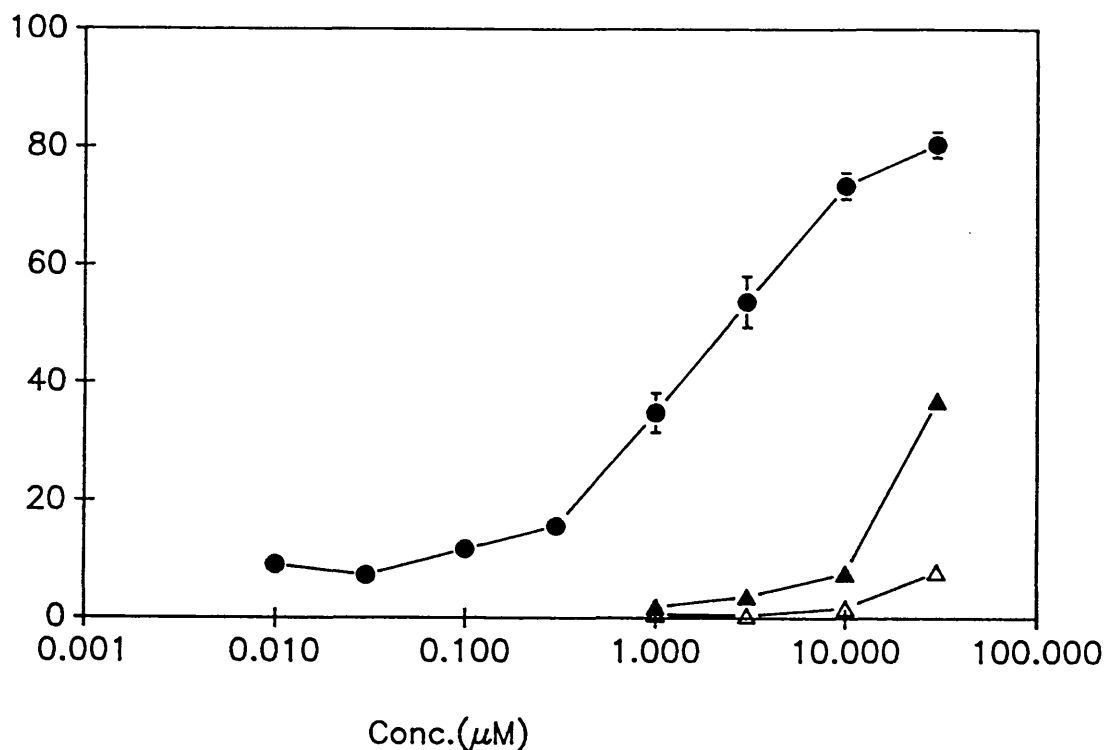


Figure 15. Dose response curves to InsP₃ (●), 6-deoxy-Ins(1,4,5)P₃ (▲) and its phosphorothioate analogue, 6-deoxy-Ins(1,4,5)PS₃ (Δ). for their ability to release ⁴⁵Ca from permeabilised rabbit platelets. Saponin permeabilised platelets were loaded with ⁴⁵Ca in the presence of mitochondrial uptake inhibitors, incubated with InsP₃ or analogue for 3 minutes at 20°C and release was determined by rapid filtration. Each point is mean ± SE of three determinations.

deoxy-2,3-cyclohexylidene 1,4,5-trisphosphate [6-deoxy-2,3-cyclohex(1,4,5)P₃] and *myo*-inositol-6-deoxy-2,3-cyclohexylidene 1,4,5-trisphosphorothioate [6-deoxy-2,3-cyclohex(1,4,5)PS₃] were tested for their ⁴⁵Ca-release ability.

6-deoxy-Ins(1,4,5)P₃ is capable of releasing small amounts of ⁴⁵Ca showing 40 ± 2% release at 30 μM, see figure 15, thus having an EC₅₀ of ≥30 μM. In comparison, its phosphorothioate analogue, 6-deoxy-Ins(1,4,5)PS₃, releases a maximum of 8 ± 1% ⁴⁵Ca at 30 μM therefore it can be said to be a very weak agonist. (n=3, mean ± SE)

<u>Compound</u>	<u>EC₅₀ at 20°C</u>
InsP ₃	1.14 ± 0.09 μM
6-deoxy-Ins(1,4,5)P ₃	≥30 μM
6-deoxy-Ins(1,4,5)PS ₃	≥30

The synthetic precursors of these two compounds follow the same pattern with the parent phosphate 6-deoxy-2,3-cyclohex-Ins(1,4,5)P₃ releasing 9 ± 1.4% at 30 μM and its phosphorothioate analogue 6-deoxy-2,3-cyclohex-Ins(1,4,5)PS₃ releasing 3 ± 1.5% at 30 μM, thus these two compounds are also very weak agonists, considering their Ca²⁺ release abilities, (n=3, mean ± SE), see figure 16.

Removal of the 6-OH of InsP₃ as shown in 6-deoxy-Ins(1,4,5)P₃ leads to a substantial loss in activity (EC₅₀ of ≥30 μM) and the substitution of the phosphate with a phosphorothioate leads to the complete loss of activity,

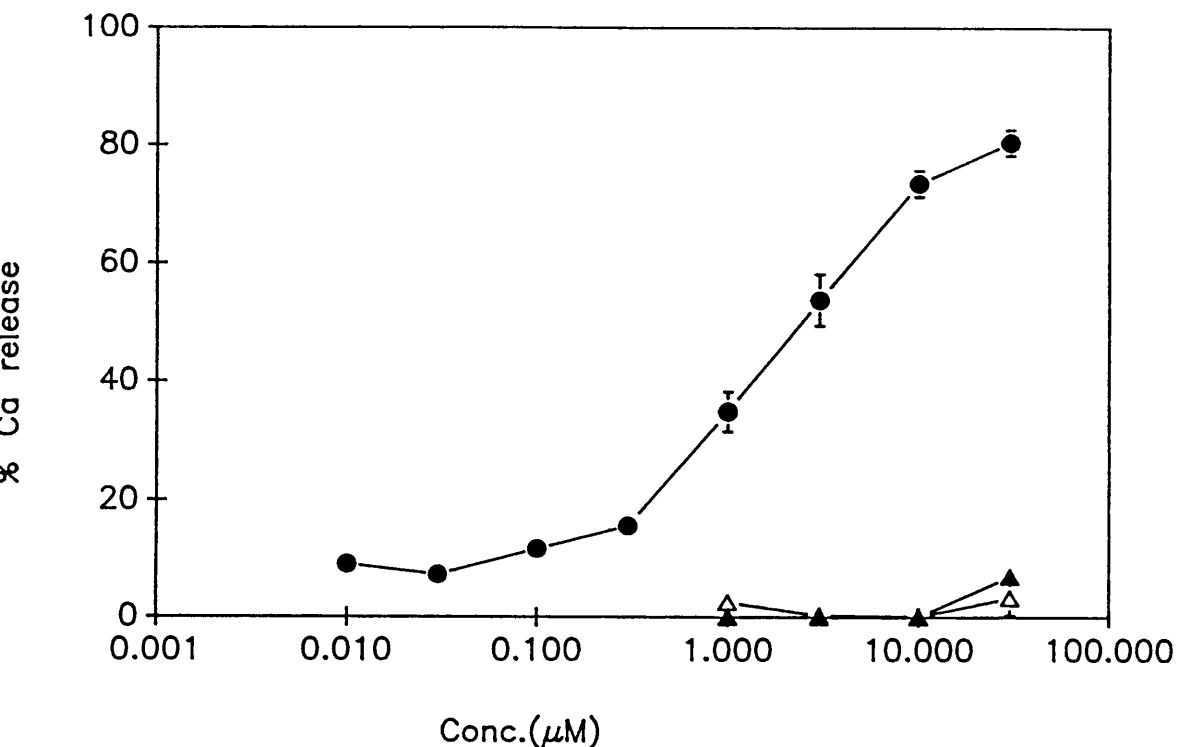


Figure 16. Dose response curves to InsP₃ (●), 6-deoxy-2,3-cyclohex-Ins(1,4,5)P₃ (▲) and its phosphorothioate analogue, 6-deoxy-2,3-cyclohex-Ins(1,4,5)PS₃ (Δ). for their ability to release ⁴⁵Ca from permeabilised rabbit platelets. Saponin permeabilised platelets were loaded with ⁴⁵Ca in the presence of mitochondrial uptake inhibitors, incubated with InsP₃ or analogue for 3 minutes at 20°C and release was determined by rapid filtration. Each point is mean ± SE of three determinations.

Compound	EC ₅₀ at 20°C
InsP ₃	1.14 ± 0.09 μM
6-deoxy-2,3-cyclohex-Ins(1,4,5)P ₃	≥ 30 μM
6-deoxy-2,3-cyclohex-Ins(1,4,5)PS ₃	≥ 30

6-deoxy-2,3-cyclohex-Ins(1,4,5)PS₃ with EC₅₀ of 30 μM. The synthetic precursors of these two analogues show no activity, and this is possibly due to the bulky cyclohexylidene group on positions 2- and 3- preventing receptor binding. Further, these compounds did not modify 3 μM InsP₃-induced ⁴⁵Ca-release, making them inactive analogues and not possessing antagonistic activity.

3.1.2.3 2-positional modification.

Myo-inositol 2,2-difluoro 1,4,5-trisphosphate [Ins(2,2)F₂(1,4,5)P₃] was tested for its ⁴⁵Ca release ability in comparison to InsP₃.

The maximal ⁴⁵Ca release induced by Ins(2,2)F₂(1,4,5)P₃ (83 ± 2.1%) was the same as that for InsP₃, this analogue having an EC₅₀ of 0.63 ± 0.09 μM compared to the EC₅₀ of InsP₃ of 1.14 ± 0.1 μM (n=3, mean ± SE), see figure 17.

Ins(2,2)F₂(1,4,5)P₃ appears two to be two fold more potent than InsP₃ at releasing ⁴⁵Ca, at 20°C and the

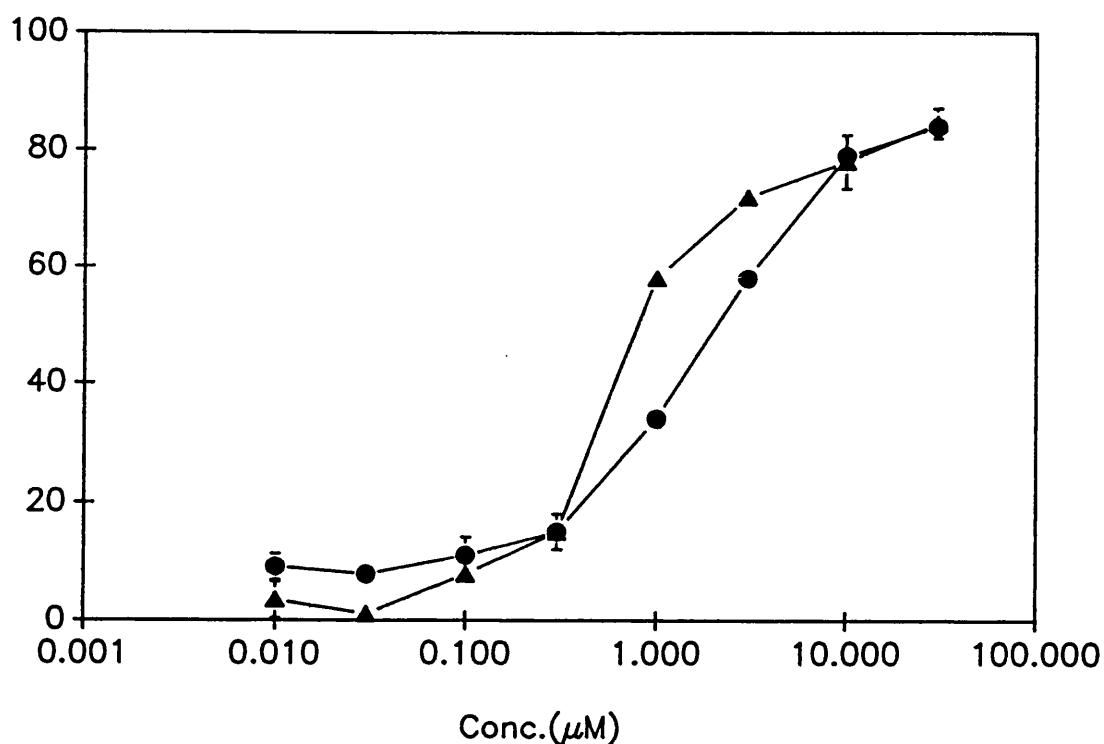


Figure 17. The dose response curves to InsP₃ (●) and Ins(2,2)F₂(1,4,5)P₃ (▲) for their ability to release ⁴⁵Ca from permeabilised rabbit platelets. Saponin permeabilised platelets were loaded with ⁴⁵Ca in the presence of mitochondrial uptake inhibitors, incubated with InsP₃ and Ins(2,2)F₂(1,4,5)P₃ for 3 minutes at 20°C and release was determined by rapid filtration. Each point is mean \pm SE of three determinations.

Compound	EC ₅₀ at 20°C
InsP ₃	1.14 ± 0.1 μM
Ins(2,2)F ₂ (1,4,5)P ₃	0.63 ± 0.09 μM

retention of ⁴⁵Ca release activity after modification at the 2-position indicates the minor role played by this 2-OH in Ca²⁺ mobilisation.

3.1.2.4 Phosphorothioate analogues of InsP₃.

Myo-inositol 1,4,5-trisphosphorothioate [Ins(1,4,5)PS₃], *myo*-inositol-1-phosphate 4,5-bisphosphorothioate [Ins(1)P(4,5)PS₂] and D/L-*myo*-inositol-1-phosphorothioate 4,5-bisphosphate [D/L-Ins(1)PS(4,5)P₂] were tested for their ⁴⁵Ca-release ability.

InsP₃ and all the above analogues were equally efficacious in their ability to release sequestered ⁴⁵Ca (figure 18, n=3, mean ± SE). However at 20°C, Ins(1)PS(4,5)P₂ was a more potent stimulus with an EC₅₀ of 0.65 ± 0.1 μM (for the racemic mixture D/L-Ins(1)PS(4,5)P₂ the EC₅₀ was 1.3 ± 0.1 μM), Ins(1,4,5)PS₃ was the next potent with an EC₅₀ of 0.8 ± 0.1 μM followed by Ins(1)P(4,5)PS₂ with an EC₅₀ of 0.85 ± 0.09 μM, see figure 18.

At 20°C, InsP₃ is the least potent with an EC₅₀ of 0.97 ± 0.11 μM (n=3), this could be due to the fact that the phosphorothioate functional groups are resistant to metabolism. Thus, it is possible that these phosphorothioate analogues are in the vicinity of the receptor for a longer period of time, increasing the likelihood

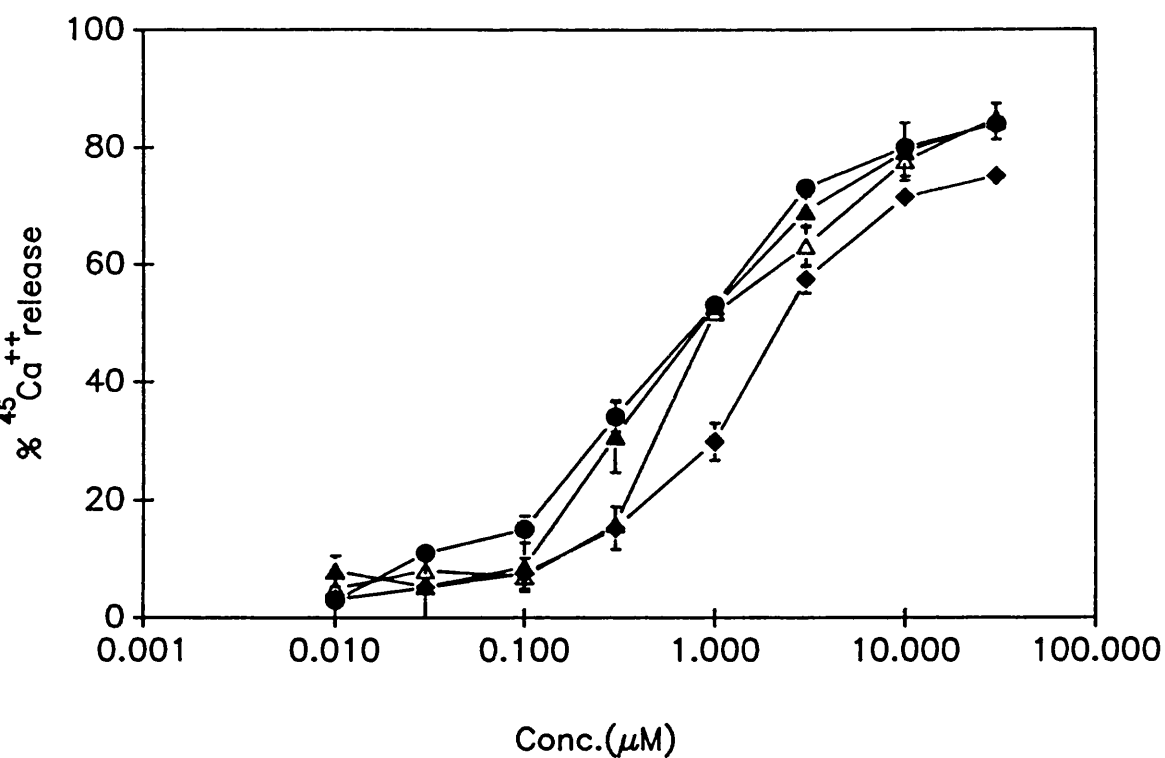


Figure 18. The effect of substituting one or more phosphate groups with phosphorothioates on the InsP_3 molecule in terms of their ability to release ^{45}Ca from permeabilised rabbit platelets. Saponin permeabilised platelets were loaded with ^{45}Ca in the presence of mitochondrial uptake inhibitors, incubated with InsP_3 (●), $\text{Ins}(1)\text{P}(4,5)\text{PS}_2$ (▲), $\text{Ins}(1)\text{PS}(4,5)\text{P}_2$ (◆) or $\text{Ins}(1,4,5)\text{PS}_3$ (Δ) for 3 minutes at 20°C and release was determined by rapid filtration. Each point is mean \pm SE of three determinations.

Compound	EC ₅₀ at 20°C
InsP ₃	0.97 ± 0.11 μM
Ins(1,4,5)PS ₃	0.8 ± 0.1 μM
Ins(1)P(4,5)PS ₂	0.85 ± 0.09 μM
D/L-Ins(1)PS(4,5)P ₂	1.3 ± 0.1 μM
[Ins(1)PS(4,5)P ₂]	0.65 ± 0.1 μM

of receptor occupation and activation. In contrast, InsP₃ is metabolised more rapidly at 20°C. Thus, is not present for long periods of time in the vicinity of the receptor and is unable to mobilise as much Ca²⁺ (see page 120).

3.1.2.5 Positional phosphate modifications of InsP₃.

D/L-Myo-inositol 1,3,4-trisphosphate [Ins(1,3,4)P₃], myo-inositol 1,3,5-trisphosphorothioate [Ins(1,3,5)PS₃] and L-myoinositol 1,4,5-trisphosphorothioate [L-Ins(1,4,5)PS₃] were tested for their Ca²⁺-mobilisation ability.

D/L-Ins(1,3,4)P₃ releases ⁴⁵Ca weakly giving 62 ± 5% at 30 μM, having an EC₅₀ ≥ 10 μM (n=3, mean ± SE). However, this analogue is considered as a full agonist as the dose response curve does not plateau but maintains an upward trend. Ins(1,3,5)PS₃ was ineffective in releasing sequestered ⁴⁵Ca with 30 μM releasing 12 ± 1.2% at 4°C. L-Ins(1,4,5)PS₃, the enantiomer of Ins(1,4,5)PS₃ (see section 3.1.2.4) was partly effective in releasing ⁴⁵Ca with 100 μM releasing 42 ± 3% of the total ⁴⁵Ca sequestered (n=3, mean ± SE),

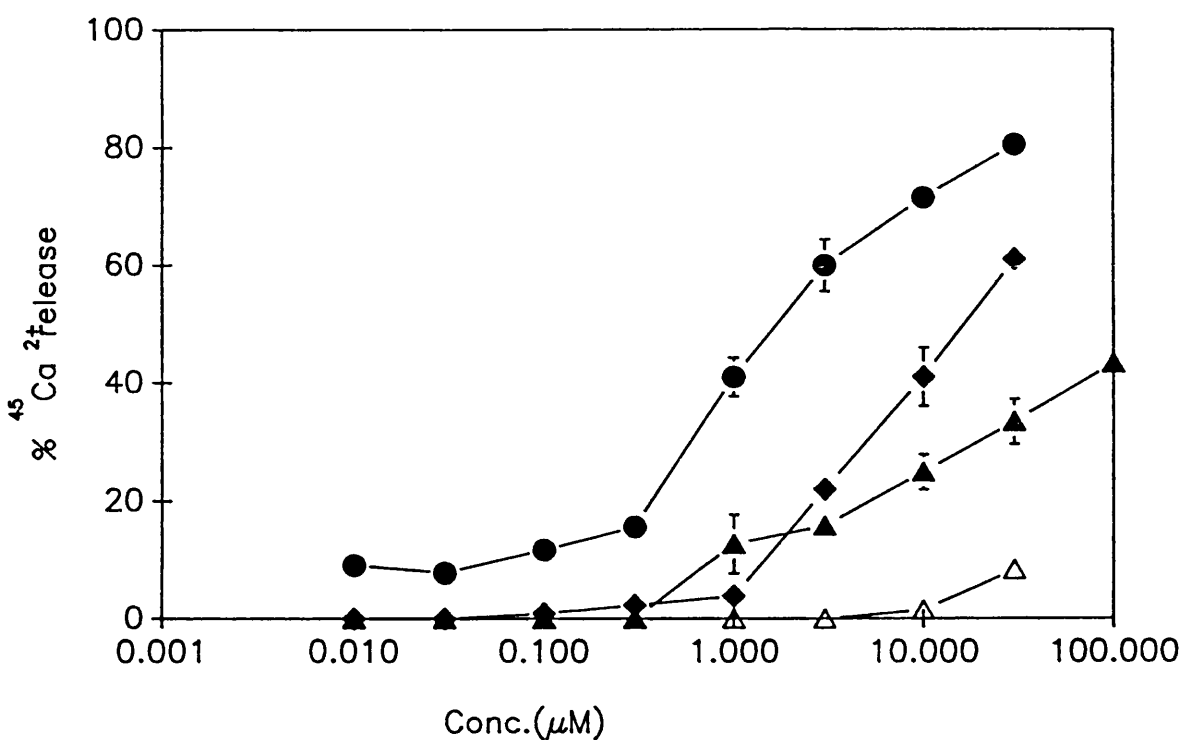


Figure 19. The ^{45}Ca release ability of some positionally modified InsP_3 (●) analogues D/L- $\text{Ins}(1,3,4)\text{P}_3$ (◆), $\text{Ins}(1,3,5)\text{PS}_3$ (△) and L- $\text{Ins}(1,4,5)\text{PS}_3$ (▲). Saponin permeabilised platelets were loaded with ^{45}Ca in the presence of mitochondrial uptake inhibitors, incubated with InsP_3 or analogue for 3 minutes at 20°C and release was determined by rapid filtration. Each point is mean \pm SE of three determinations.

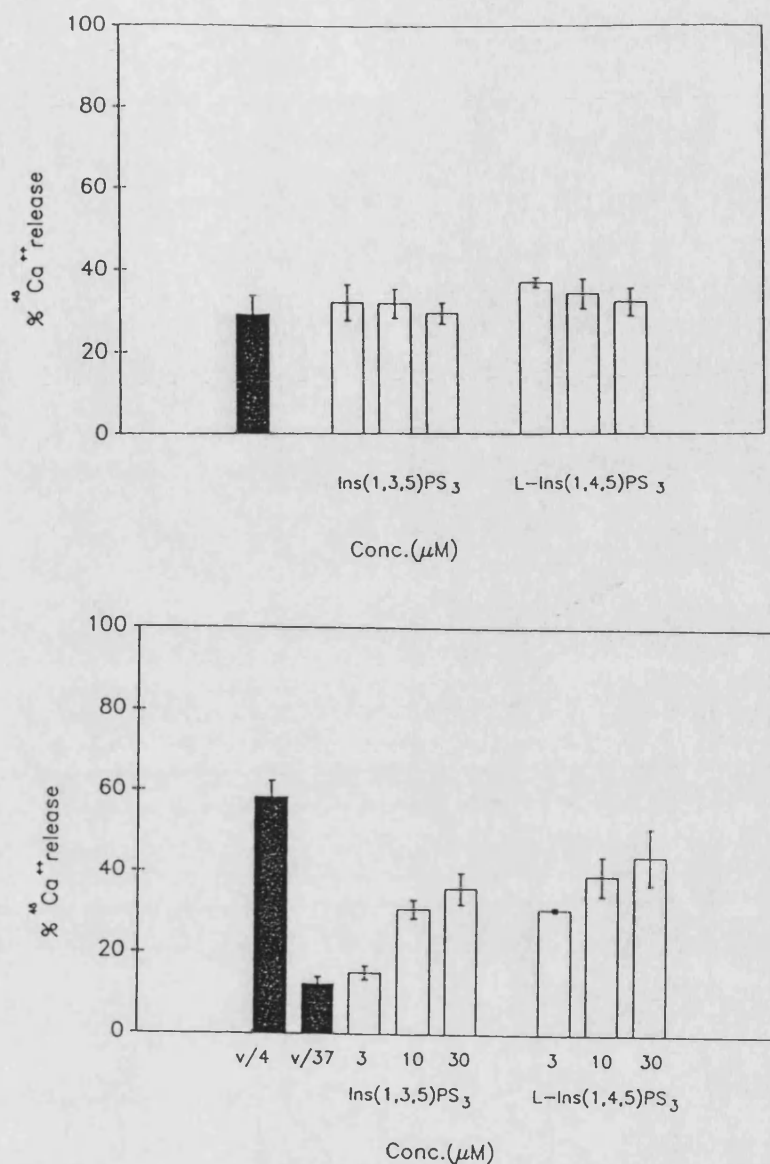


Figure 20. The effect of the metabolic enzyme inhibitors, L-Ins(1,4,5)PS₃ and Ins(1,3,5)PS₃, on InsP₃-stimulated ⁴⁵Ca-release at (a) 4°C and (b) 37°C. Saponin permeabilised platelets were loaded with ⁴⁵Ca in the presence of mitochondrial uptake inhibitors and co-incubated with a dose range of the analogue (open bars) or vehicle (closed bars) at 4°C (v/4) or 37°C (v/37) and InsP₃ (100nM at 4°C and 30μM at 37°C) for 3 minutes, v/20 is control at 20°C, and release was determined by rapid filtration. Each point is mean ± SE of three determinations.

see figure 19. This analogue is a full agonist, although this may be due to the potentially small presence of contaminating D-isomer in this compound.

Compound	EC ₅₀ at 4°C
InsP ₃	0.18 ± 0.1 μM
D/L-Ins(1,3,4)P ₃	≥ 30 μM
Ins(1,3,5)PS ₃	≥ 100 μM
L-Ins(1,4,5)PS ₃	≥ 30 μM

In addition, D/L-Ins(1,3,4)P₃ did not inhibit InsP₃-induced ⁴⁵Ca-release at 4°C or 20°C thus showing no antagonistic effects. In contrast to this, Ins(1,3,5)PS₃ and L-Ins(1,4,5)PS₃ did potentiate 3 μM InsP₃-induced ⁴⁵Ca-release at 37°C but had no effect on 100 nM InsP₃-induced ⁴⁵Ca release at 4°C, indicating that these compounds affect the metabolism of InsP₃, see figure 20.

This is supported by later data (see section on metabolism) where these two compounds were found to be InsP₃ 5-phosphatase inhibitors.

3.1.2.6 Miscellaneous inositol phosphate analogues.

Scyllo-inositol 1,2,4-trisphosphate [*scyllo*-Ins(1,2,4)P₃], *myo*-inositol-4-methylene carboxylate 3,5-bisphosphate [Ins(4)CO(3,5)P₂] and *myo*-inositol-1-phosphate 4,5-pyrophosphate [Ins(1)P(4,5)pyrophosphate] were examined for their Ca²⁺-release ability in comparison to InsP₃.

Scyllo-Ins(1,2,4)P₃ (figure 21) is equally efficacious as InsP₃

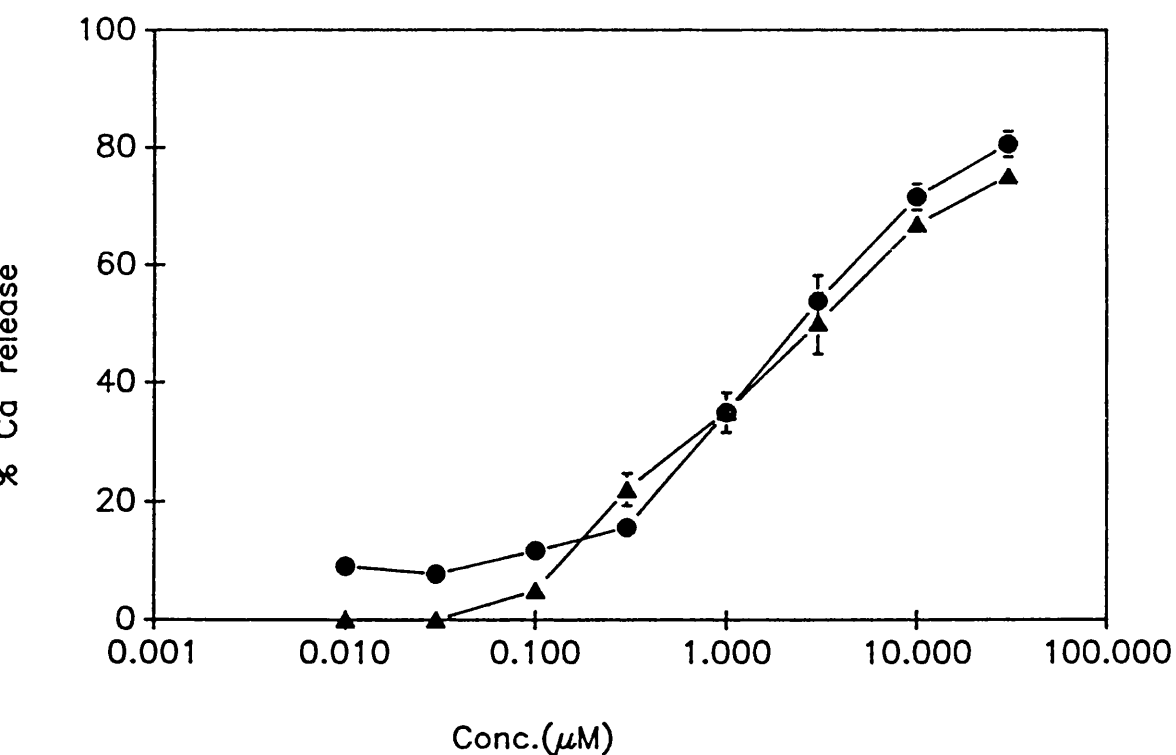


Figure 21. The dose response curve to InsP₃ (●) and *scyllo*-Ins(1,2,4)P₃ (▲) for their ability to release ⁴⁵Ca from permeabilised platelets. Saponin permeabilised platelets were loaded with ⁴⁵Ca in the presence of mitochondrial uptake inhibitors, incubated with a InsP₃ or analogue for 3 minutes at 20°C and release was determined by rapid filtration. Each point is mean ± SE of three determinations.

in releasing sequestered ^{45}Ca at 20°C , giving a maximal release of $78 \pm 3\%$ with an EC_{50} of $0.94 \pm 0.07 \mu\text{M}$ ($n=3$, mean \pm SE).

Compound	EC_{50} at 20°C
InsP_3	$1.1 \pm 0.1 \mu\text{M}$
<i>scyllo</i> - $\text{Ins}(1,2,4)\text{P}_3$	$0.94 \pm 0.07 \mu\text{M}$

$\text{Ins}(4)\text{CO}(3,5)\text{P}_2$ and $\text{Ins}(1)\text{P}(4,5)$ pyrophosphate did not have the capacity to release ^{45}Ca , furthermore neither analogue inhibited InsP_3 -induced ^{45}Ca release at 20°C . $\text{Ins}(4)\text{CO}(3,5)\text{P}_2$ does not possess a phosphate group on the 1-position, in addition, it has a phosphate on the 3-position and a bulky methylene carboxylate on the 4-position rather than a phosphate group, all of these factors could contribute to the lack of activity. As for $\text{Ins}(1)\text{P}(4,5)$ pyrophosphate, the correct positions are occupied but the 4,5-vicinal phosphates which are deemed essential for activity are coupled together to form a pyrophosphate system, which in principle could mimic the conformation adopted by InsP_3 in chelating a metal ion at the 4,5-bisphosphate.

3.1.2.7 Myo-inositol 1,3,4,5-tetrakisphosphate.

$[\text{Ins}(1,3,4,5)\text{P}_4]$ and a phosphorothioate analogue, *myo*-inositol-3-phosphorothioate 1,4,5-trisphosphate $[\text{Ins}(3) \text{PS}(1,4,5)\text{P}_3]$ were tested for their Ca^{2+} -release ability.

$\text{Ins}(1,3,4,5)\text{P}_4$ is a naturally occurring metabolite of InsP_3 and releases ^{45}Ca with a lower potency than InsP_3 ,

giving $54 \pm 2\%$ ^{45}Ca release at $30\mu\text{M}$ and an EC_{50} of $\geq 10\mu\text{M}$ ($n=3$, mean \pm SE). This implies that Ca^{2+} release upon InsP_3 -stimulation can be attributed in the main to InsP_3 with $\text{Ins}(1,3,4,5)\text{P}_4$ playing a very minor role, see figure 22.

Compound	EC_{50} at 20°C
InsP_3	$1.15 \pm 0.1\mu\text{M}$
$\text{Ins}(1,3,4,5)\text{P}_4$	$\geq 10\mu\text{M}$
$\text{Ins}(3)\text{PS}(1,4,5)\text{P}_3$	$\geq 10\mu\text{M}$

$\text{Ins}(3)\text{PS}(1,4,5)\text{P}_3$, a phosphorothioate analogue of $\text{Ins}(1,3,4,5)\text{P}_4$, initially synthesised as an inhibitor of InsP_3 3-kinase, is also weak in releasing ^{45}Ca , giving $51 \pm 1\%$ release at $30\mu\text{M}$ and an EC_{50} of $\geq 10\mu\text{M}$ ($n=3$, mean \pm SE). The ^{45}Ca release profiles of the two compounds are different in that although low doses of $\text{Ins}(1,3,4,5)\text{P}_4$ do not release ^{45}Ca , at $3\mu\text{M}$ and up to $30\mu\text{M}$ a rapid elevation is observed in % release (figure 22). In contrast, $\text{Ins}(3)\text{PS}(1,4,5)\text{P}_3$ is weak at releasing ^{45}Ca and only slowly rises to reach $51 \pm 1\%$, close to that of $\text{Ins}(1,3,4,5)\text{P}_4$.

3.1.2.8 Inositol tetrakisphosphate analogues.

Myo-inositol 1,2,4,5-tetrakisphosphate [$\text{Ins}(1,2,4,5)\text{P}_4$] and its synthetic precursor *myo*-inositol-3,6-dibenzoyl 1,2,4,5-tetrakisphosphate [$\text{Ins}(3,6)\text{Bz}_2(1,2,4,5)\text{P}_4$] were examined for their Ca^{2+} -release ability in comparison to InsP_3 .

$\text{Ins}(1,2,4,5)\text{P}_4$ is a full agonist, as potent as InsP_3 in releasing ^{45}Ca ,

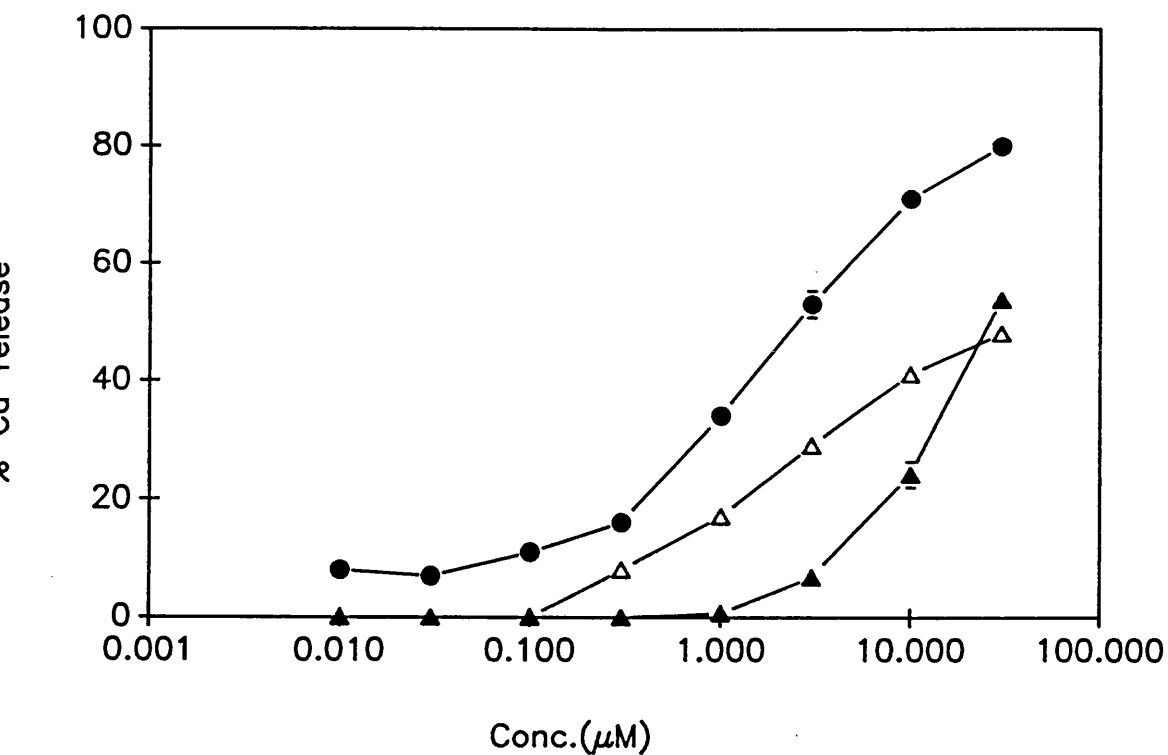


Figure 22. The dose response curve to InsP₃ (●), its natural metabolite Ins(1,3,4,5)P₄ (▲) and its phosphorothioate analogue Ins(3)PS(1,4,5)P₃ (Δ) for their ability to release ⁴⁵Ca from permeabilised platelets. Saponin permeabilised platelets were loaded with ⁴⁵Ca in the presence of mitochondrial uptake inhibitors, incubated with a InsP₃ or analogue for 3 minutes at 20°C and release was determined by rapid filtration. Each point is mean ± SE of three determinations.

giving maximal release of $72 \pm 5\%$ at $30\mu\text{M}$ with an EC_{50} of $0.53 \pm 0.04\mu\text{M}$ ($n=3$, mean \pm SE), this is in contrast to $\text{Ins}(1,3,4,5)\text{P}_4$ which is weak at releasing ^{45}Ca , see figure 23.

Compound	EC_{50} at 20°C
InsP_3	$1.1 \pm 0.09\mu\text{M}$
$\text{Ins}(1,3,4,5)\text{P}_4$	$\geq 10\mu\text{M}$
$\text{Ins}(1,2,4,5)\text{P}_4$	$0.53 \pm 0.04\mu\text{M}$
$\text{Ins}(3,6)\text{Bz}_2(1,2,4,5)\text{P}_4$	$\geq 30\mu\text{M}$

$\text{Ins}(3,6)\text{Bz}_2(1,2,4,5)\text{P}_4$, the synthetic precursor of $\text{Ins}(1,2,4,5)\text{P}_4$ was completely inactive in releasing ^{45}Ca . This could be due to steric hindrance caused by the presence of the large benzoyl groups on the 3- and 6-positions, however more likely is the occupation of the 6-OH which is known to be essential for activity (see later).

3.1.2.9 Inositol tetrakisphosphate analogues.

Myo-inositol 1,3,4,6-tetrakisphosphate [$\text{Ins}(1,3,4,6)\text{P}_4$] and its phosphorothioate analogue, *myo*-inositol 1,3,4,6-tetrakisphosphate [$\text{Ins}(1,3,4,6)\text{PS}_4$] were examined for their Ca^{2+} -release ability in comparison to InsP_3 .

$\text{Ins}(1,3,4,6)\text{P}_4$ releases sequestered ^{45}Ca from permeabilised platelets with a lower potency than InsP_3 , releasing $58 \pm 3\%$ Ca^{2+} at $30\mu\text{M}$ in comparison to $\text{Ins}(1,3,4,5)\text{P}_4$ which releases $54 \pm 2\%$ Ca^{2+} (at 20°C). The phosphorothioate

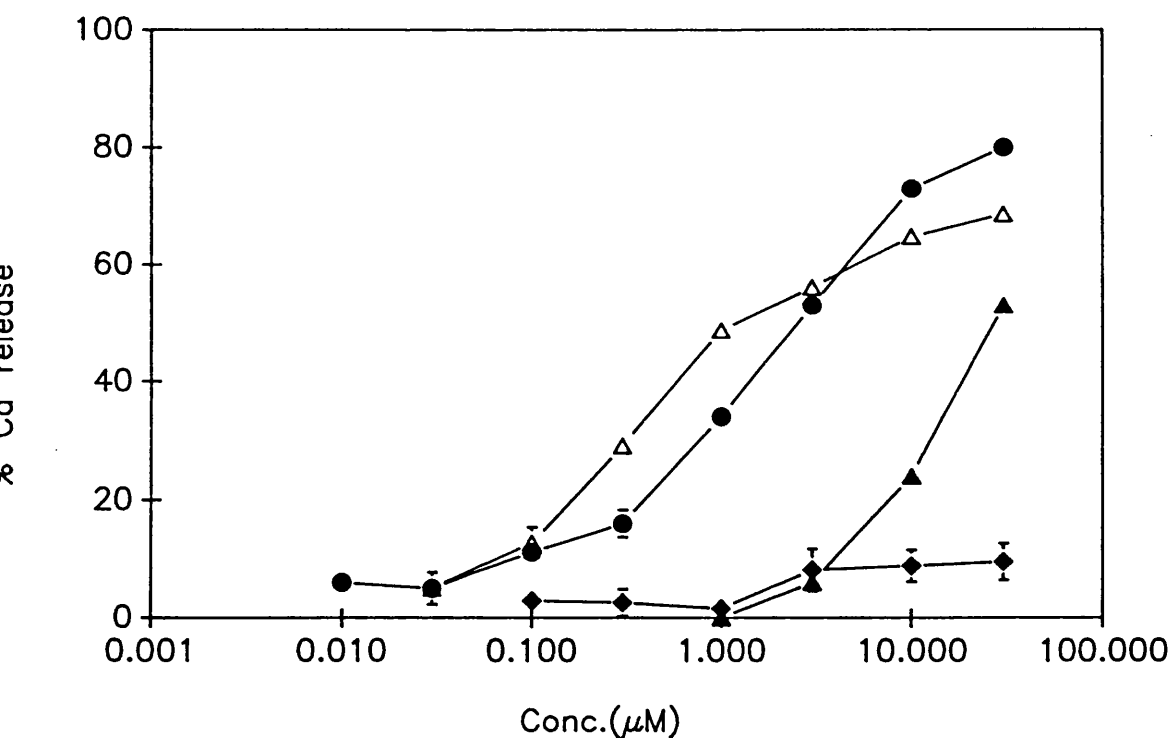


Figure 23. The dose response curve to InsP₃ (●), Ins(1,3,4,5)P₄ (▲) and some Ins(1,3,4,5)P₄ analogues, Ins(1,2,4,5)P₄ (Δ) and Ins(3,6)BZ₂(1,2,4,5)P₄ (◆) for their ability to release ⁴⁵Ca from permeabilised platelets. Saponin permeabilised platelets were loaded with ⁴⁵Ca in the presence of mitochondrial uptake inhibitors, incubated with a InsP₃ or analogue for 3 minutes at 20°C and release was determined by rapid filtration. Each point is mean ± SE of three determinations.

analogue Ins(1,3,4,6)PS₄, is weaker than its parent phosphate and 100 μ M releases $49 \pm 4\%$ Ca²⁺, see figure 24.

Compound	EC ₅₀ at 4°C
InsP ₃	$0.18 \pm 0.1 \mu\text{M}$
Ins(1,3,4,6)P ₄	$\geq 10 \mu\text{M}$
Ins(1,3,4,6)PS ₄	$\geq 30 \mu\text{M}$

3.1.3 Partial agonists.

3.1.3.1 *Scyllo*-inositol 1,2,4,5-tetrakisphosphate [*scyllo*-Ins(1,2,4,5)P₄] and its phosphorothioate analogue *scyllo*-inositol 1,2,4,5-tetrakisphorothioate [*scyllo*-Ins(1,2,4,5)PS₄] were examined for their Ca²⁺-release ability in comparison to InsP₃.

Scyllo-Ins(1,2,4,5)P₄ is a full agonist releasing 85% of the sequestered ⁴⁵Ca and appearing to be more efficacious than InsP₃ having an EC₅₀ of $0.1 \pm 0.05 \mu\text{M}$ in comparison to InsP₃ which gives a maximal release of 79% and an EC₅₀ of $1.14 \pm 0.19 \mu\text{M}$, see figure 25 (n=3, mean \pm SE).

The phosphorothioate analogue *scyllo*-Ins(1,2,4,5)PS₄ exhibited a low intrinsic activity, releasing a maximal of $61.7 \pm 11\%$ of the total ⁴⁵Ca, having only 78% of the intrinsic activity or efficacy of InsP₃. This indicates that this analogue,

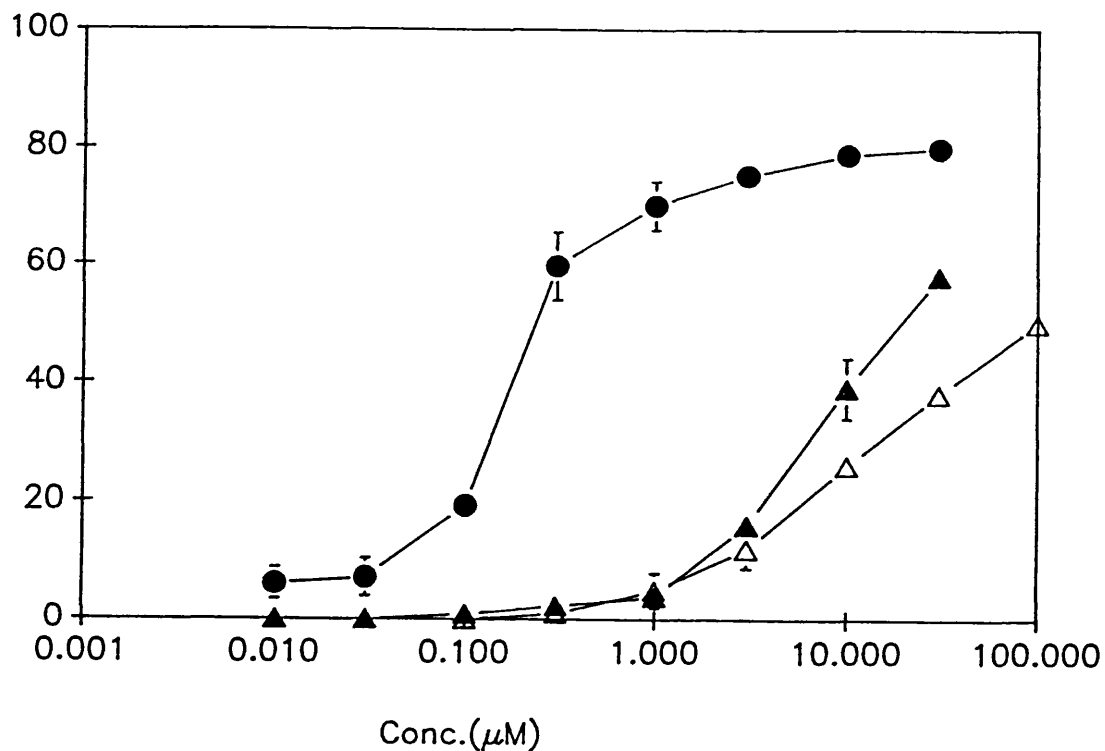


Figure 24. The dose response curve to InsP₃ (●) and some naturally occurring inositol phosphates Ins(1,3,4,6)P₄ (▲) and its phosphorothioate analogue Ins(1,3,4,6)PS₄ (△) for their ability to release ⁴⁵Ca from permeabilised platelets. Saponin permeabilised platelets were loaded with ⁴⁵Ca in the presence of mitochondrial uptake inhibitors, incubated with a InsP₃ or analogue for 3 minutes at 4°C and release was determined by rapid filtration. Each point is mean \pm SE of three determinations.

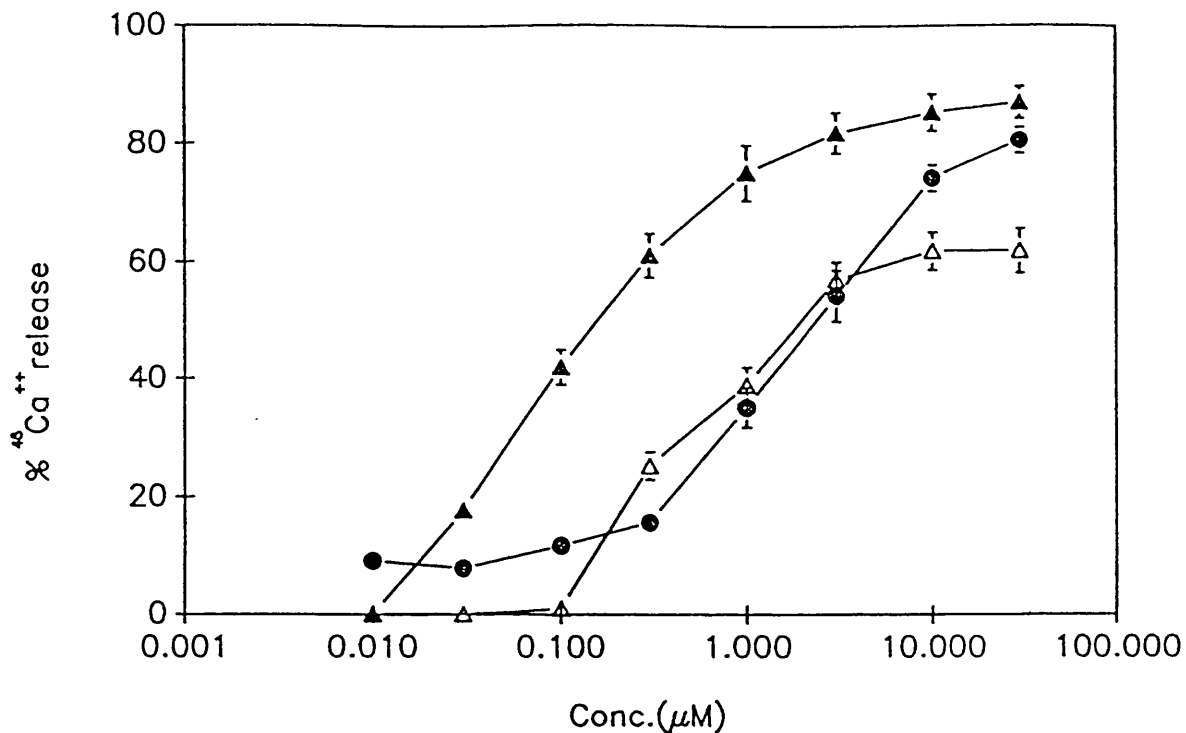


Figure 25. The dose response curve to InsP₃ (●), *scyllo*-Ins(1,2,4,5)P₄ (▲) and its phosphorothioate analogue *scyllo*-Ins(1,2,4,5)PS₄ (Δ) for their ability to release ⁴⁵Ca from permeabilised platelets. Saponin permeabilised platelets were loaded with ⁴⁵Ca in the presence of mitochondrial uptake inhibitors, incubated with a InsP₃ or analogue for 3 minutes at 20°C and release was determined by rapid filtration. Each point is mean ± SE of three determinations.

Compound	EC ₅₀ at 20°C
InsP ₃	1.14 ± 0.2 μM
<i>Scyllo</i> -Ins(1,2,4,5)P ₄	0.1 ± 0.05 μM
<i>Scyllo</i> -Ins(1,2,4,5)PS ₄	≥ 30 μM

scyllo-Ins(1,2,4,5)PS₄, is a partial agonist. However, *scyllo*-Ins(1,2,4,5)PS₄ did not inhibit InsP₃-induced ⁴⁵Ca-release at 20°C.

3.1.3.2 *L-chiro*-inositol 2,3,5-trisphosphate [*L-chiro*-Ins(2,3,5)P₃] and its phosphorothioate analogue *L-chiro*-inositol 2,3,5-trisphosphorothioate [*L-chiro*-Ins(2,3,5)PS₃] were examined for their Ca²⁺-release ability in comparison to InsP₃.

L-chiro-Ins(2,3,5)P₃ is a full agonist releasing 82% of the loaded ⁴⁵Ca and having an EC₅₀ of 1.9 ± 0.1 μM compared to InsP₃ [EC₅₀ of 1.04 ± 0.08 μM and a maximal release of 80%, see figure 26 (n=3, mean ± SE)].

Compound	EC ₅₀ at 20°C
InsP ₃	1.04 ± 0.08 μM
<i>L-chiro</i> -Ins(2,3,5)P ₃	1.9 ± 0.1 μM
<i>L-chiro</i> -Ins(2,3,5)PS ₃	≥ 30 μM

L-chiro-Ins(2,3,5)PS₃, a phosphorothioate analogue, displayed low intrinsic activity, giving maximal ⁴⁵Ca release of 42.3 ± 6%, thus having 52%

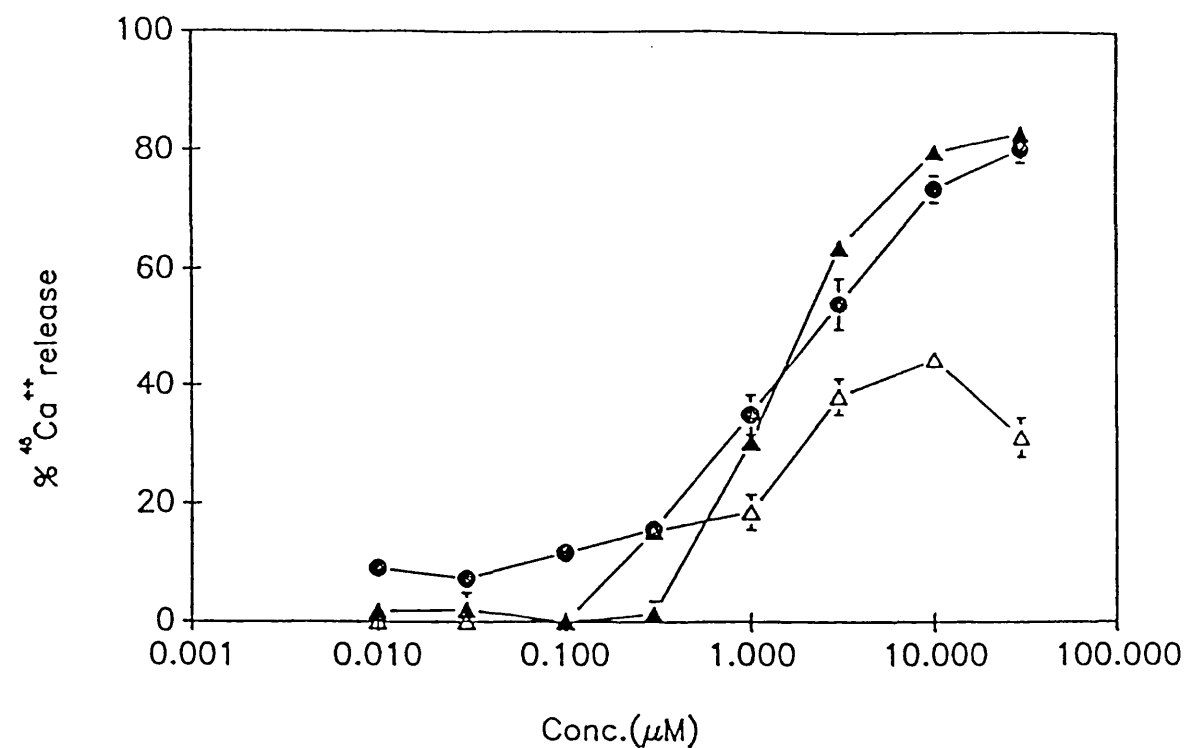


Figure 26. The dose response curve to InsP₃ (●), L-chiro-Ins(2,3,5)P₃ (▲) and its phosphorothioate analogue L-chiro-Ins(2,3,5)PS₃ (Δ) for their ability to release ^{45}Ca from permeabilised platelets. Saponin permeabilised platelets were loaded with ^{45}Ca in the presence of mitochondrial uptake inhibitors, incubated with a InsP₃ or analogue for 3 minutes at 20°C and release was determined by rapid filtration. Each point is mean \pm SE of three determinations.

of the activity of InsP_3 and hence is a partial agonist. Further, *L-chiro*- $\text{Ins}(2,3,5)\text{PS}_3$ did not produce an inhibition of InsP_3 -induced ^{45}Ca -release.

3.1.3.3 *Myo*-inositol 1,4,6-trisphosphate [$\text{Ins}(1,4,6)\text{P}_3$] and its phosphorothioate analogue *myo*-inositol 1,4,6-trisphosphorothioate [$\text{Ins}(1,4,6)\text{PS}_3$] were examined for their Ca^{2+} -release ability in comparison to InsP_3 .

$\text{Ins}(1,4,6)\text{P}_3$ is a weak full agonist giving a maximal release of 75% at $30\mu\text{M}$, with an EC_{50} of $2.07 \pm 0.08\mu\text{M}$ compared to InsP_3 which has an EC_{50} of $0.18 \pm 0.04\mu\text{M}$ and a maximal release of 82%, see figure 27 ($n=3$, mean \pm SE).

<u>Compound</u>	<u>EC_{50} at 4°C</u>
InsP_3	$0.18 \pm 0.04\mu\text{M}$
$\text{Ins}(1,4,6)\text{P}_3$	$2.07 \pm 0.08\mu\text{M}$
$\text{Ins}(1,4,6)\text{PS}_3$	$\geq 30\mu\text{M}$

Upon phosphate modification to a phosphorothioate to give $\text{Ins}(1,4,6)\text{PS}_3$, a substantial loss in activity was observed. $\text{Ins}(1,4,6)\text{PS}_3$ gives a maximal ^{45}Ca release of $21.1\% \pm 0.4\%$, having 25% of the intrinsic activity of InsP_3 , thus is a partial agonist.

In contrast to two partial agonists above, *L-chiro*- $\text{Ins}(2,3,5)\text{PS}_3$ *scyllo*- $\text{Ins}(1,2,4,5)\text{PS}_4$ tested for inhibitory effects on InsP_3 -induced ^{45}Ca -release, $\text{Ins}(1,4,6)\text{PS}_3$ produces a dose dependent inhibition of 300nM InsP_3 -induced ^{45}Ca release, such that $100\mu\text{M}$

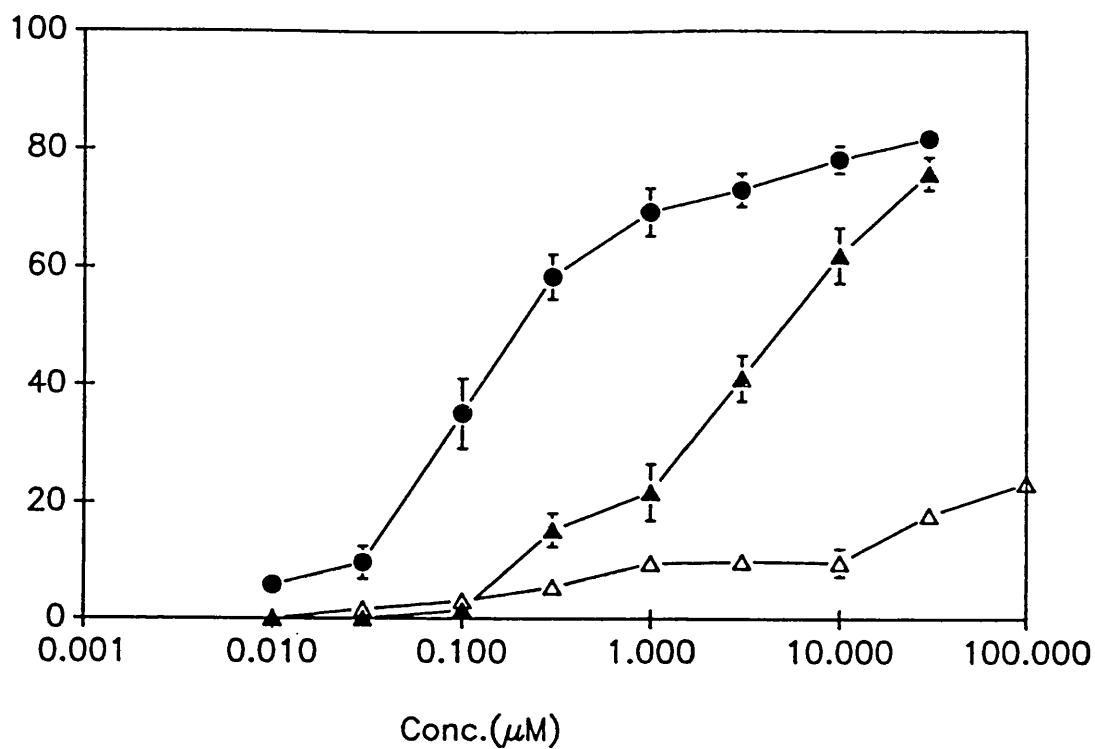


Figure 27. The dose response curve to InsP₃ (●), Ins(1,4,6)P₃ (▲) and its phosphorothioate analogue Ins(1,4,6)PS₃ (Δ) for their ability to release ⁴⁵Ca from permeabilised platelets. Saponin permeabilised platelets were loaded with ⁴⁵Ca in the presence of mitochondrial uptake inhibitors, incubated with a InsP₃ or analogue for 3 minutes at 4°C and release was determined by rapid filtration. Each point is mean \pm SE of three determinations.

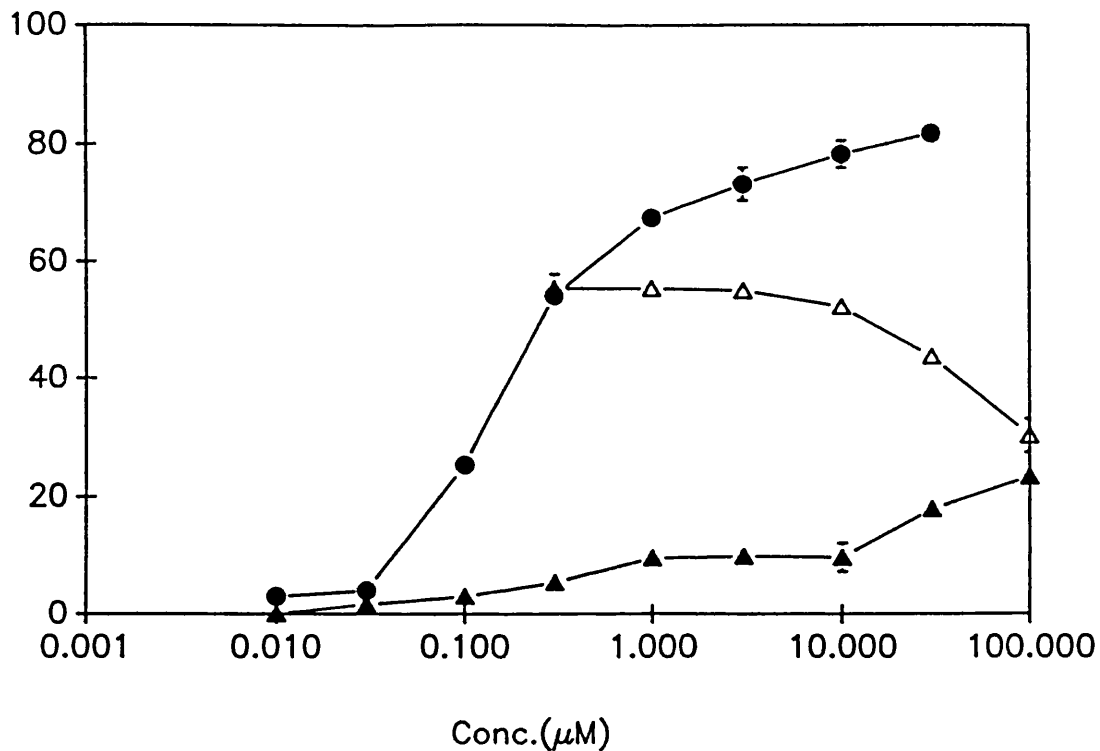


Figure 28. The dose response curve to InsP₃ (●) and Ins(1,4,6)PS₃ (▲) for their ability to release ⁴⁵Ca from permeabilised platelets and the effect of Ins(1,4,6)PS₃ on InsP₃-induced ⁴⁵Ca release (Δ). Saponin permeabilised platelets were loaded with ⁴⁵Ca in the presence of mitochondrial uptake inhibitors, inhibition of InsP₃-induced ⁴⁵Ca release was observed upon co-incubations of Ins(1,4,6)PS₃ (300nM- 100μM) with 300nM InsP₃ at 4°C in a dose dependent manner such that 100μM Ins(1,4,6)PS₃ produced ≈ 50% inhibition. Each point is mean ± SE of three determinations.

Ins(1,4,6)P₃ produced approximately 50% inhibition, see figure 28.

3.1.4 Inhibitors of InsP₃-induced ⁴⁵Ca-release.

3.1.4.1 The effect of heparin on InsP₃-induced ⁴⁵Ca-release.

Heparin (100ng/ml- 100µg/ml) did not induce ⁴⁵Ca-release from permeabilised platelets loaded with ⁴⁵Ca, in addition, heparin (100ng/ml- 100µg/ml) incubated with permeabilised platelets for 5, 10 and 30 minutes did not induce inhibition of 3µM InsP₃ induced ⁴⁵Ca-release at 20°C, see figure 29. Further, heparin of lower molecular weight (3000) did not cause any inhibition of InsP₃-induced Ca²⁺ mobilisation.

3.1.4.2 The effect of Decavanadate on InsP₃-induced ⁴⁵Ca-release.

Decavanadate inhibits 3µM InsP₃-induced ⁴⁵Ca-release at 20°C, with an IC₅₀ of $7.82 \pm 0.4 \mu\text{M}$ (n=3, mean \pm SE), see figure 30. Sodium orthovanadate, the precursor of decavanadate was inactive, having no impact on InsP₃-induced Ca²⁺-mobilisation.

3.1.4.3 The effect of Benzene 1,2,4-trisphosphate on InsP₃-induced ⁴⁵Ca-release.

Benzene 1,2,4-trisphosphate (1- 500µM) did not produce an inhibition of 3µM InsP₃ induced ⁴⁵Ca-release at 20°C, in addition, benzene 1,2,4-trisphosphate (1- 100µM) did not produce the inhibition of 300nM InsP₃ induced ⁴⁵Ca-release at 4°C, see figure 31.

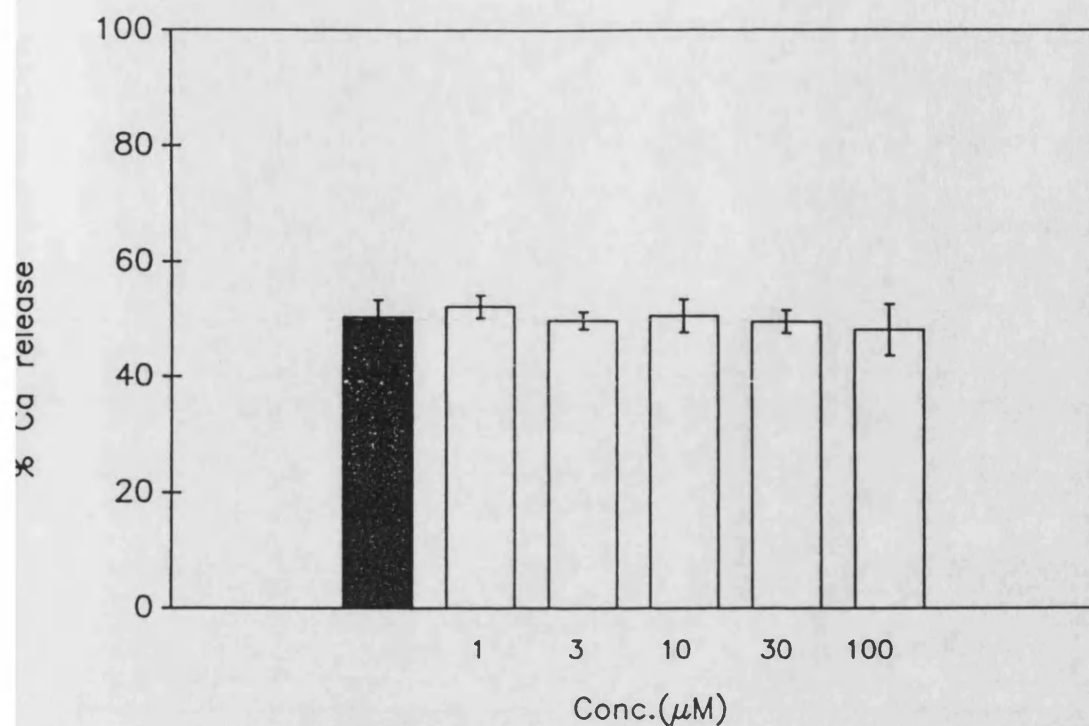


Figure 29. The effect of heparin on InsP_3 -induced ^{45}Ca release. Saponin permeabilised platelets were loaded with ^{45}Ca in the presence of mitochondrial uptake inhibitors, co-incubations of vehicle (closed bars), or heparin, 300nM-100 μM (open bars), with 300nM InsP_3 at 4°C. Each point is mean \pm SE of three determinations.

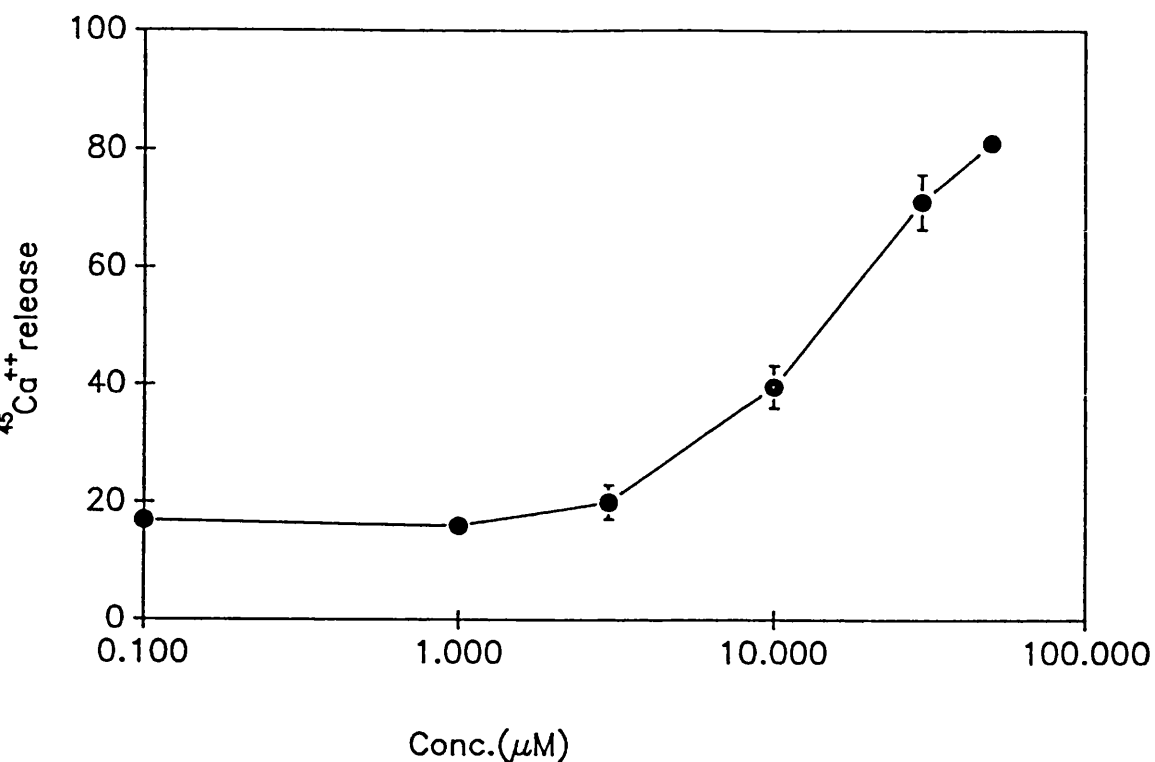


Figure 30. The inhibition of 3μM InsP₃-induced ⁴⁵Ca-release at 20°C by a dose range of decavanadate. Saponin permeabilised platelets were loaded with ⁴⁵Ca in the presence of mitochondrial uptake inhibitors, were incubated with decavanadate (0.1- 30μM) for 3 minutes and subsequently challenged with 3μM InsP₃ for 3 minutes. Each point is mean ± SE of three determinations.

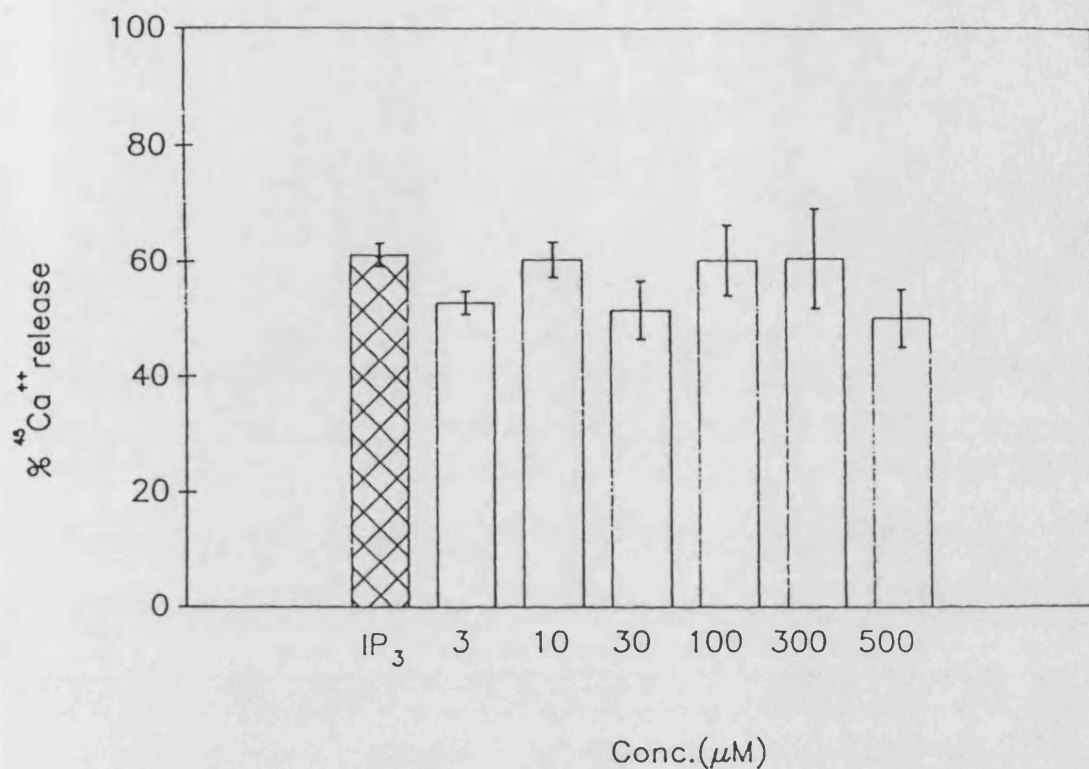


Figure 31. The effect of benzene trisphosphate on InsP_3 -induced ^{45}Ca release. Saponin permeabilised platelets were loaded with ^{45}Ca in the presence of mitochondrial uptake inhibitors, co-incubations of vehicle (closed bars), or benzene trisphosphate, 300nM- 500 μM (open bars) with 300nM InsP_3 at 4°C. Each point is mean \pm SE of three determinations.

3.1.5 The effect of temperature on InsP₃-induced Ca²⁺ release.

Dose response curves for InsP₃-induced ⁴⁵Ca release determined at 4°C, 20°C and 37°C show a shift of the response to the right with increasing temperature. At 4°C, a full dose-response curve is observed with an EC₅₀ of 0.18 ± 0.1 μM (n=6, mean ± SE), but this is reduced at 20°C having an EC₅₀ of 1.14 ± 0.2 μM (n=6, mean ± SE), and by 37°C the lower concentrations tested (10nM- 1 μM) release no Ca²⁺, and the remaining curve is very shallow (EC₅₀ ≥ 10 μM), see figure 32 (n=6, mean ± SE).

<u>Temperature</u>	<u>EC₅₀</u>
4°C	0.18 ± 0.1 μM
20°C	1.14 ± 0.2 μM
37°C	≥ 10 μM

3.1.6 The effect of temperature on *scyllo*-Ins(1,2,4,5)P₄-induced ⁴⁵Ca release.

scyllo-Ins(1,2,4,5)P₄ dose response curves at 4°C and 20°C are identical, giving maximal release of 90 ± 3% ⁴⁵Ca with an EC₅₀ of 0.1 ± 0.05 μM (n=3, mean ± SE) whereas at 37°C, the EC₅₀ is reduced to 5.1 ± 0.2 μM with 30 μM giving 66 ± 2.5% ⁴⁵Ca release, see figure 33.

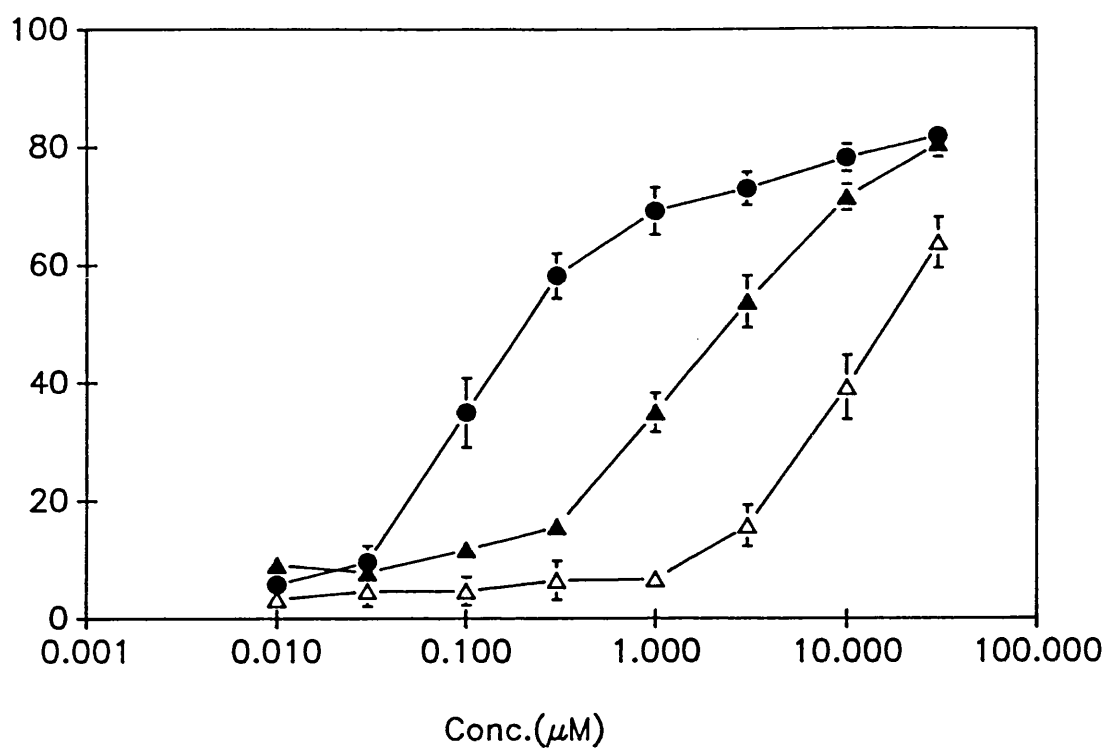


Figure 32. The effect of temperature on InsP₃-induced ⁴⁵Ca release. Saponin permeabilised platelets were loaded with ⁴⁵Ca in the presence of mitochondrial uptake inhibitors and challenged with InsP₃ for 3 minutes at 4°C (●), 20°C (▲) and 37°C (Δ) and release was determined by rapid filtration. Each point is mean ± SE of three determinations.

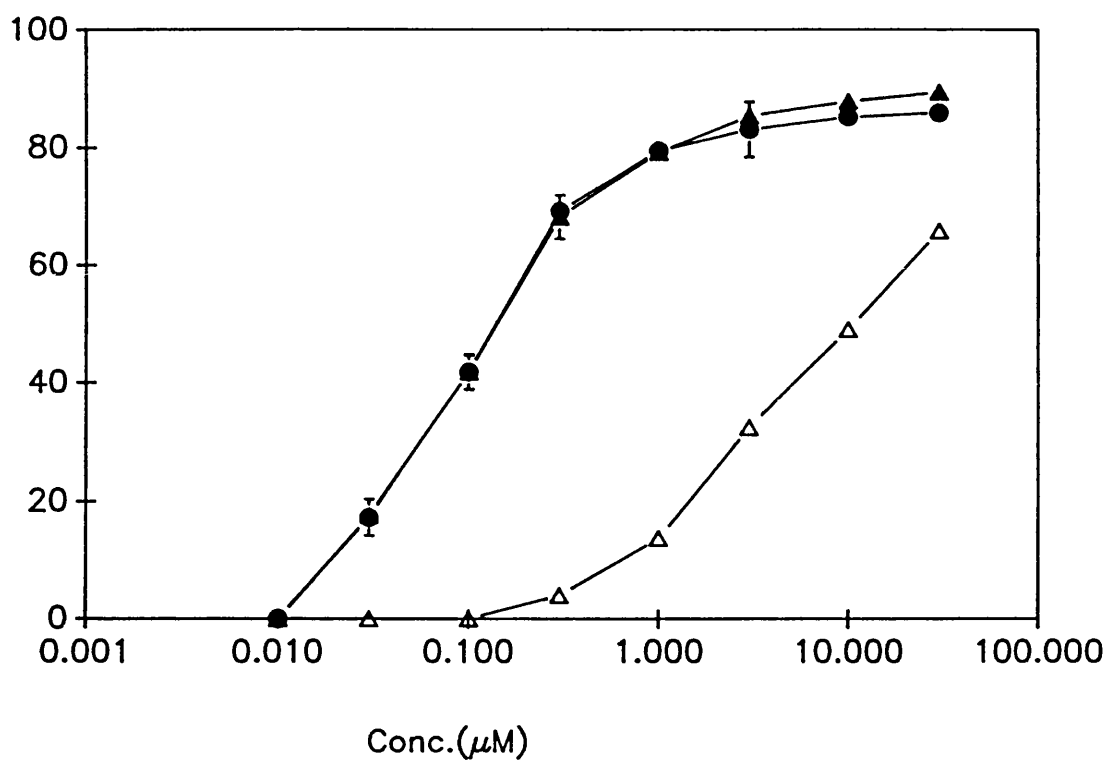


Figure 33. The effect of temperature on *scyllo*-Ins(1,2,4,5)P₄-induced ⁴⁵Ca release. Saponin permeabilised platelets were loaded with ⁴⁵Ca in the presence of mitochondrial uptake inhibitors and challenged with *scyllo*-Ins(1,2,4,5)P₄ for 3 minutes at 4°C (●), 20°C (▲) and 37°C (△) and release was determined by rapid filtration. Each point is mean \pm SE of three determinations.

<u>Temperature</u>	<u>EC₅₀</u>
4°C	0.1 ± 0.05 μM
20°C	0.1 ± 0.05 μM
37°C	5.1 ± 0.2 μM

3.1.7 ⁴⁵Ca release by non-inositol phosphate compounds.

3.1.7.1 ⁴⁵Ca-release ability of cyclic ADP-ribose (cADPR), the endogenous activator of the ryanodine receptor.

cADPR (100nM- 3 μM) did not elicit ⁴⁵Ca-release in permeabilised platelets at 20°C. In addition, ADP-ribose (ADPR), is also ineffective in mobilising Ca²⁺.

3.1.7.2 ⁴⁵Ca-release by the direct activation of protein kinase C by phorbol esters and diacylglycerol (DAG).

Phorbol myristate acetate, PMA, (1- 300nM) and DAG (3- 300nM) did not mobilise ⁴⁵Ca at 4°C or 20°C, in addition, both PMA and DAG did not attenuate or enhance 300nM or 3 μM InsP₃-induced ⁴⁵Ca release at 4°C and 20°C respectively.

3.2 [^3H]InsP₃ metabolism by the 5-phosphatase and the 3-kinase enzymes at 4°C, 20°C and 37°C, and the effect of inositol phosphate analogues on InsP₃ metabolism.

3.2.1 Investigation of [^3H]InsP₃ metabolism in permeabilised platelets at 4°C, 20°C and 37°C.

[^3H]InsP₃ metabolism by the two enzymes, the 5-phosphatase and the 3-kinase was examined, as shown in section 3.1.5, InsP₃-induced ^{45}Ca release changes with temperature, InsP₃ being more potent in releasing ^{45}Ca at 4°C than at 37°C. The EC₅₀ of InsP₃-induced ^{45}Ca release can be seen on page 12017.

This loss in potency is attributed to metabolism. In order to confirm this hypothesis, InsP₃ metabolism at the three temperatures, 4°C, 20°C and 37°C was investigated.

The rate of [^3H]InsP₃ metabolism was found to be temperature dependent and the order of the rate of metabolism was 37°C > 20°C > 4°C, figures 34, 35 and 36.

At 4°C, InsP₃ metabolism is very slow and by 3 minutes, over 70% of InsP₃ remains, at 10 minutes the major metabolite present being Ins(1,4)P₂ and overall very little Ins(1,3,4,5)P₄ is produced ($\approx 5\%$), see figure 34.

At 20°C a very different profile can be seen. By 1 minute, a substantial amount of metabolism has occurred, giving rise to Ins(1,4)P₂ and Ins(1,3,4,5)P₄; Ins(1,3,4)P₃ is produced from Ins(1,3,4,5)P₄ at 3 minutes and the major metabolite at this time is Ins(1,4)P₂. At 10 minutes little InsP₃ remains, the major metabolites being Ins(1,3,4)P₃ and InsP₁.

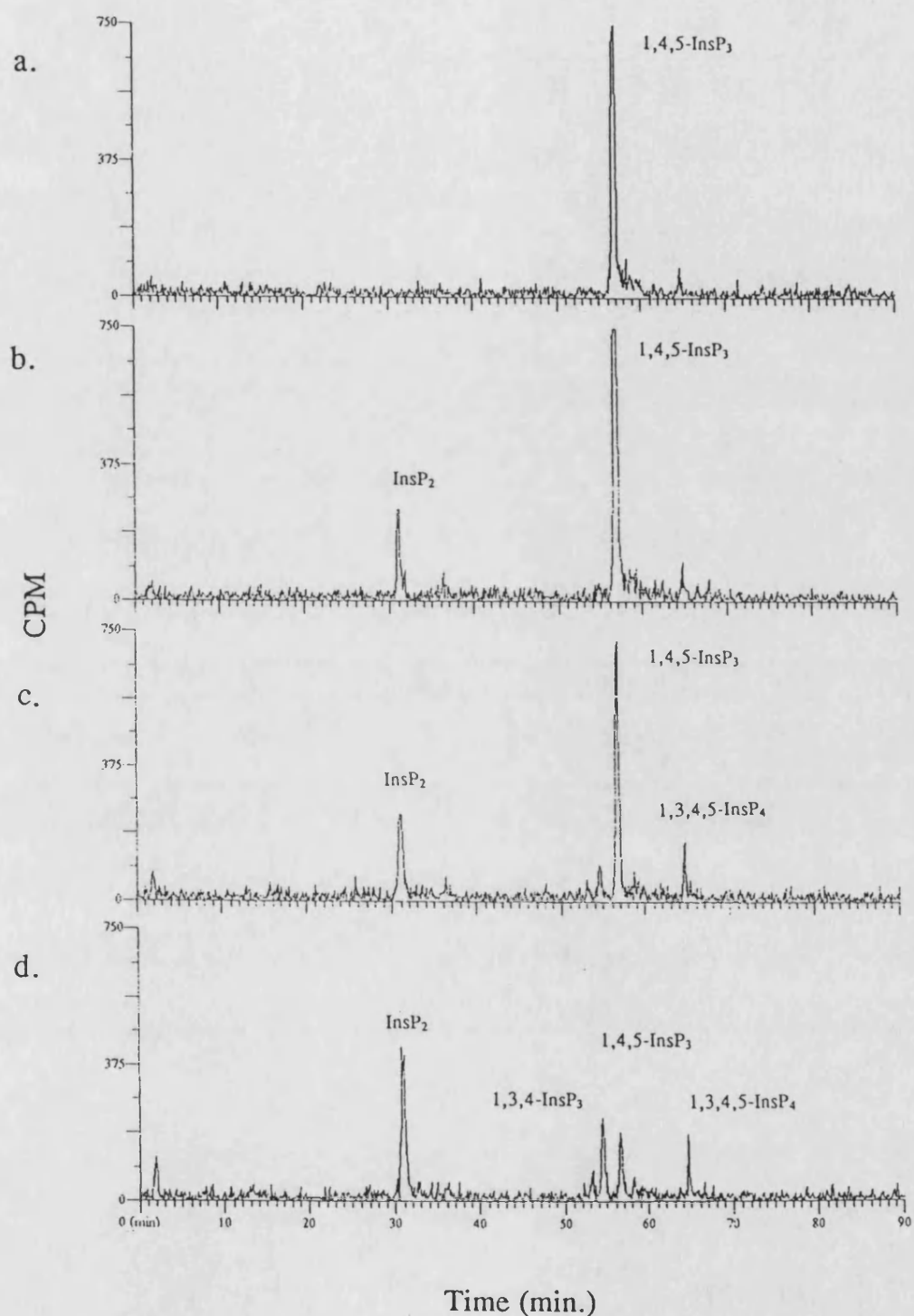


Figure 34. HPLC separation of $[^3\text{H}]\text{InsP}_3$ metabolites at 4°C . $100\mu\text{l}$ Platelets were challenged with $[^3\text{H}]\text{InsP}_3$ at 4°C and metabolism was terminated at 0, 1, 3 and 10 minutes. The phosphates were then extracted and separated on an anion exchange column. (a) $[^3\text{H}]\text{InsP}_3$ standard; (b) 1 min.; (c) 3 min.; (d) 10 minutes. Traces are representative of three separate experiments.

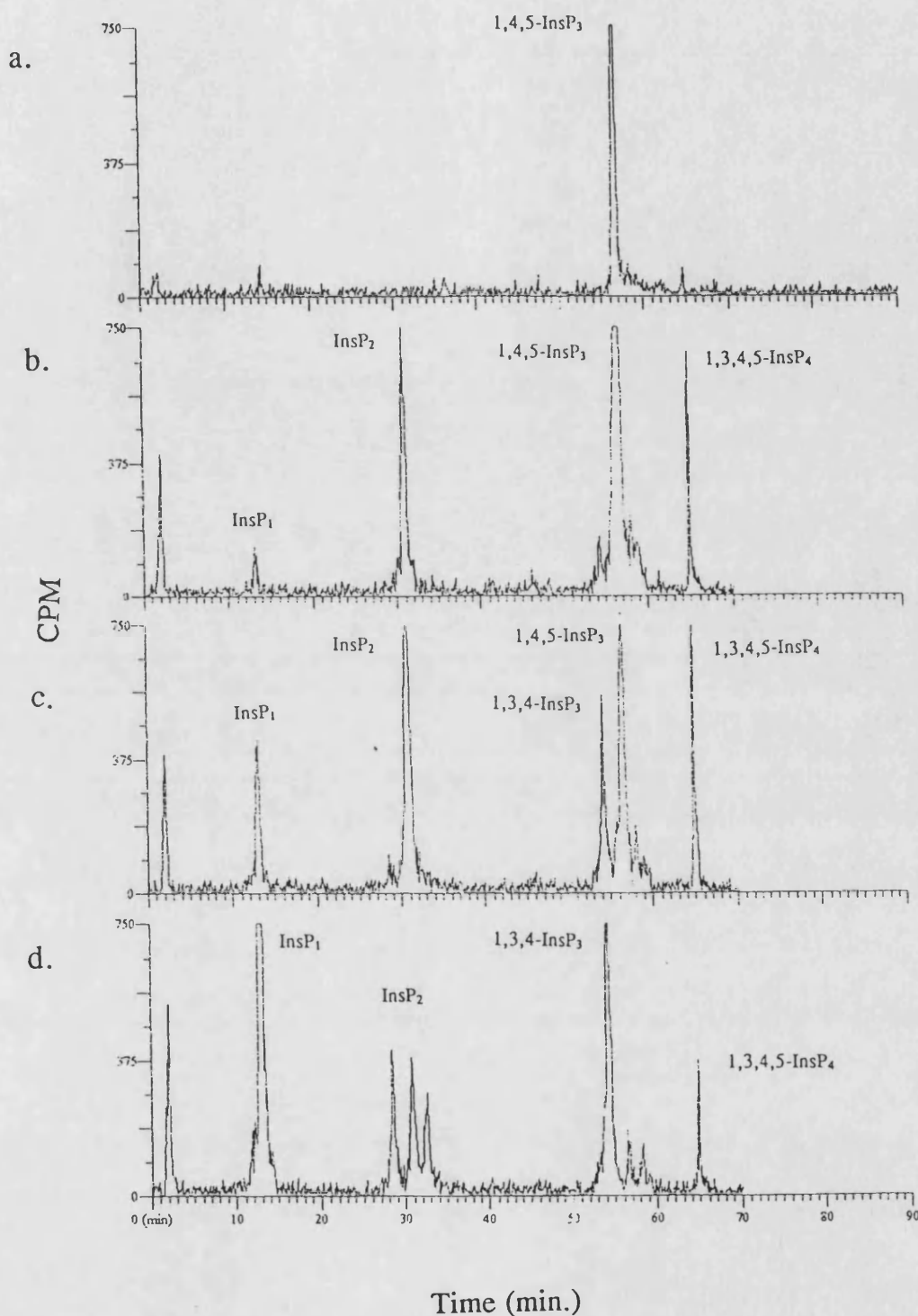


Figure 35. HPLC separation of $[^3\text{H}]\text{InsP}_3$ metabolites at 20°C . $100\mu\text{l}$ samples of platelets were challenged with $[^3\text{H}]\text{InsP}_3$ at 20°C and metabolism was terminated at 0, 1, 3 and 10 minutes. The phosphates were then extracted and separated on an anion exchange column. (a) $[^3\text{H}]\text{InsP}_3$ standard; (b) 1 min.; (c) 3 min.; (d) 10 minutes. Traces are representative of three separate experiments.

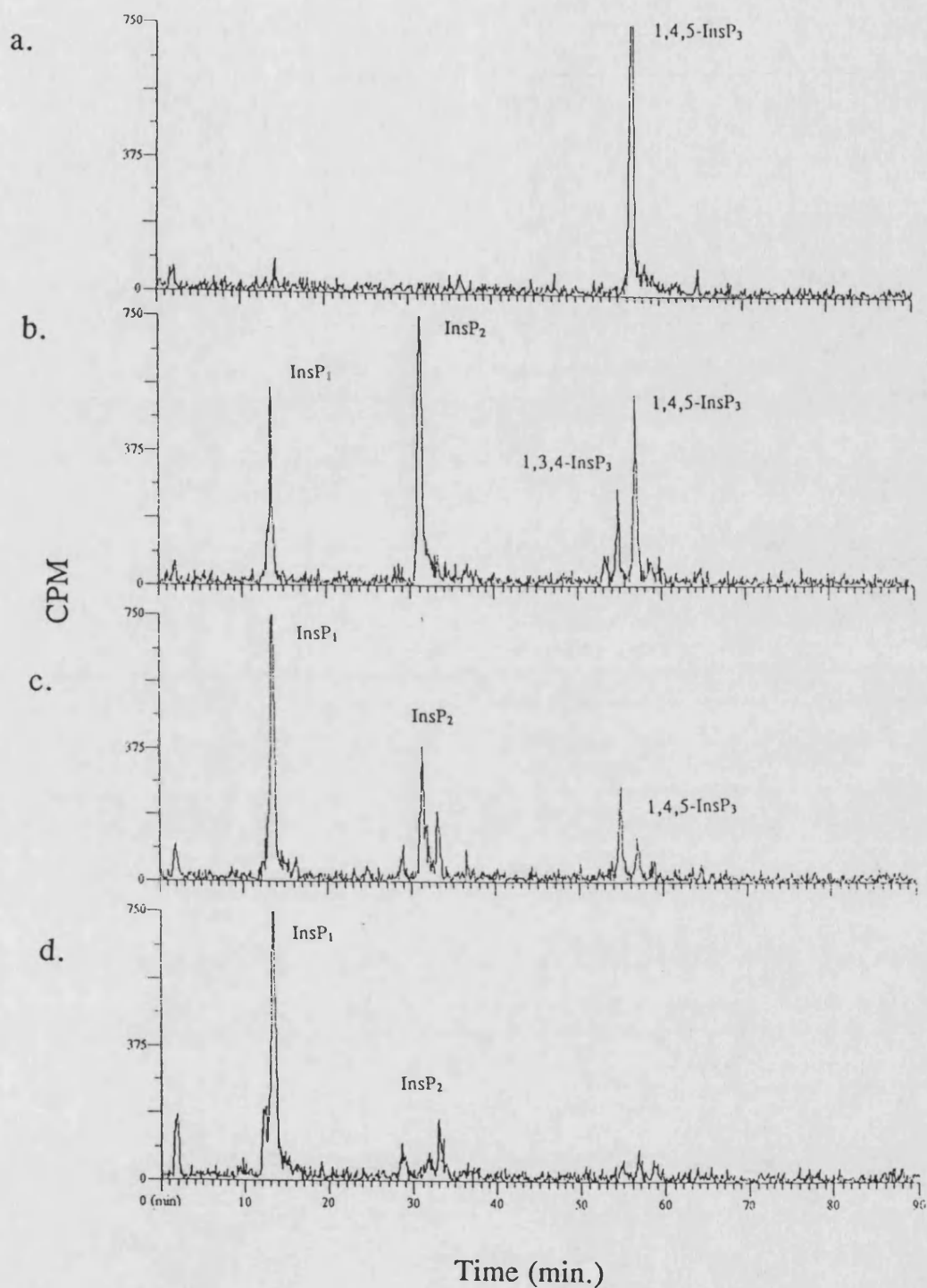


Figure 36. HPLC separation of [^3H]InsP₃ metabolites at 37°C. 100 μl samples of platelets were challenged with [^3H]InsP₃ at 37°C and metabolism was terminated at 0, 1, 3 and 10 minutes. The phosphates were then extracted and separated on an anion exchange column. (a) [^3H]InsP₃ standard; (b) 1 min.; (c) 3 min.; (d) 10 minutes. Traces are representative of three separate experiments.

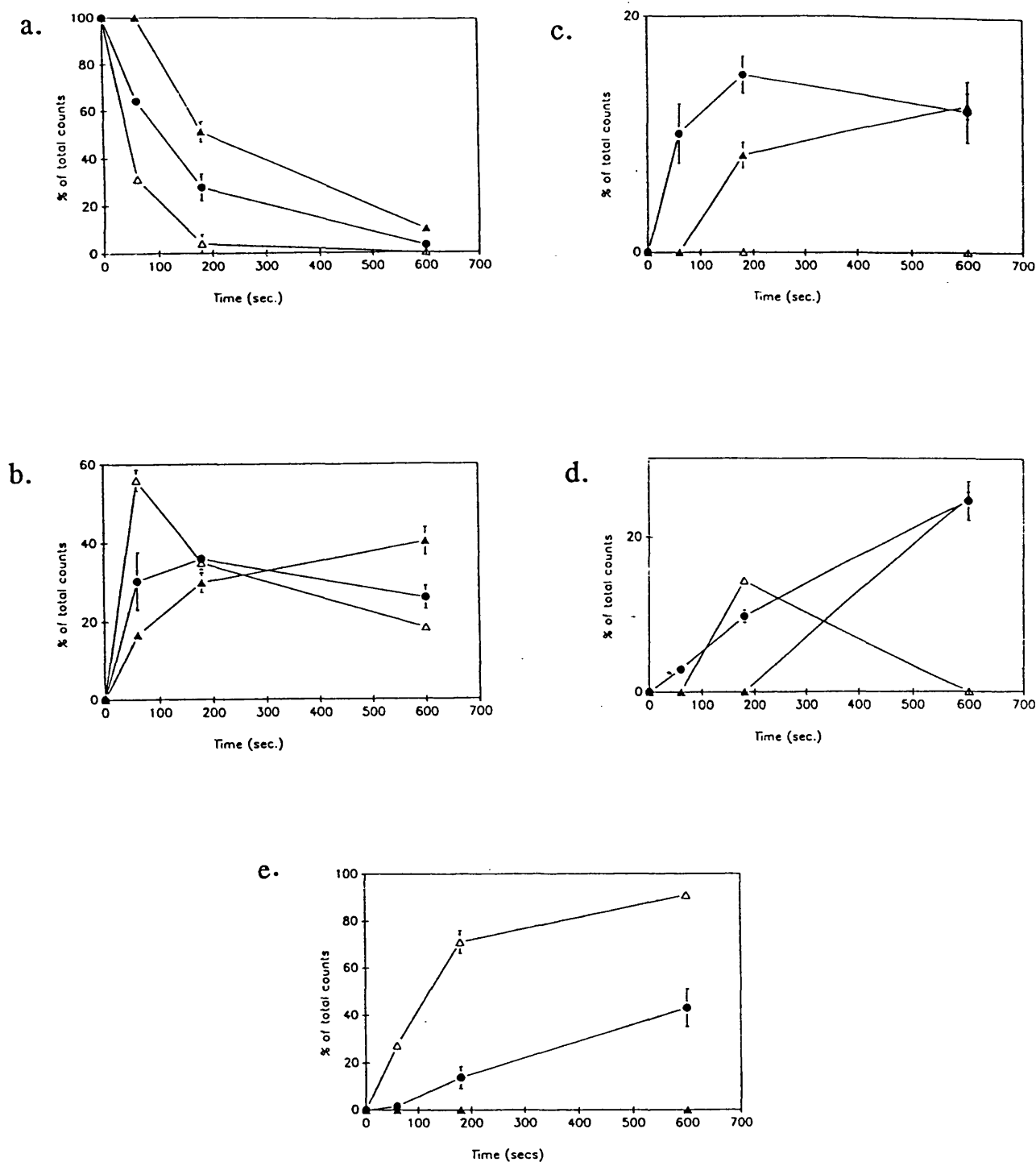


Figure 37. The effect of temperature 4°C (▲), 20°C (●) and 37°C (△) on (a) loss of $[^3\text{H}]\text{InsP}_3$; (b) InsP_2 accumulation; (c) $\text{Ins}(1,3,4,5)\text{P}_4$ accumulation (d) $\text{Ins}(1,3,4)\text{P}_3$ accumulation, and (e) InsP_1 accumulation. Each point is mean \pm SE of three determinations.

However at 37°C [^3H]InsP₃ is metabolised very rapidly with only 20% [^3H]InsP₃ remaining at 1 minute, the major metabolite at this time being Ins(1,4)P₂, a peak corresponding to Ins(1,3,4)P₃ is present but it only constitutes 10% of the total counts, and at 10 minutes InsP₁ is the only metabolite left. Thus metabolism at the three temperatures follows very different metabolic patterns and at 4° and 37°C very little Ins(1,3,4,5)P₄ and hence little Ins(1,3,4)P₃ is produced.

Thus for any subsequent metabolism studies, platelets were maintained at 20°C as this shows the greatest range of metabolites over the 10 minute span.

3.2.2 The effect of inositol phosphate analogues on [^3H]InsP₃ metabolism.

Two inositol phosphate analogues, L-Ins(1,4,5)PS₃ and Ins(1,3,5)PS₃ have been shown in a preliminary fashion to have an effect on the metabolism of [^3H]nsP₃, see figure 20. This inhibitory activity that was investigated in this study. InsP₃ metabolism is complex and proceeds via two enzymes a 5-phosphatase and a 3-kinase. These two compounds can be used as tools to elucidate the significance of enzyme inhibition and the possibility of isozymes.

An inhibition of InsP₃ metabolism by both L-Ins(1,4,5)PS₃ and Ins(1,3,5)PS₃ was observed, being maximal at 10 minutes, see figures 38, 39 and 40. The effect of each analogue on inositol phosphate metabolism and production is shown in figure 41 and 42. From figure 41 it can be seen that L-Ins(1,4,5)PS₃ produces a dose dependent conservation of InsP₃ (compared to control), thus inhibiting InsP₃ metabolism; it produces a reduction in Ins(1,4)P₂ production (compared to control), hence inhibiting Ins(1,4)P₂ formation from InsP₃ via the 5-phosphatase

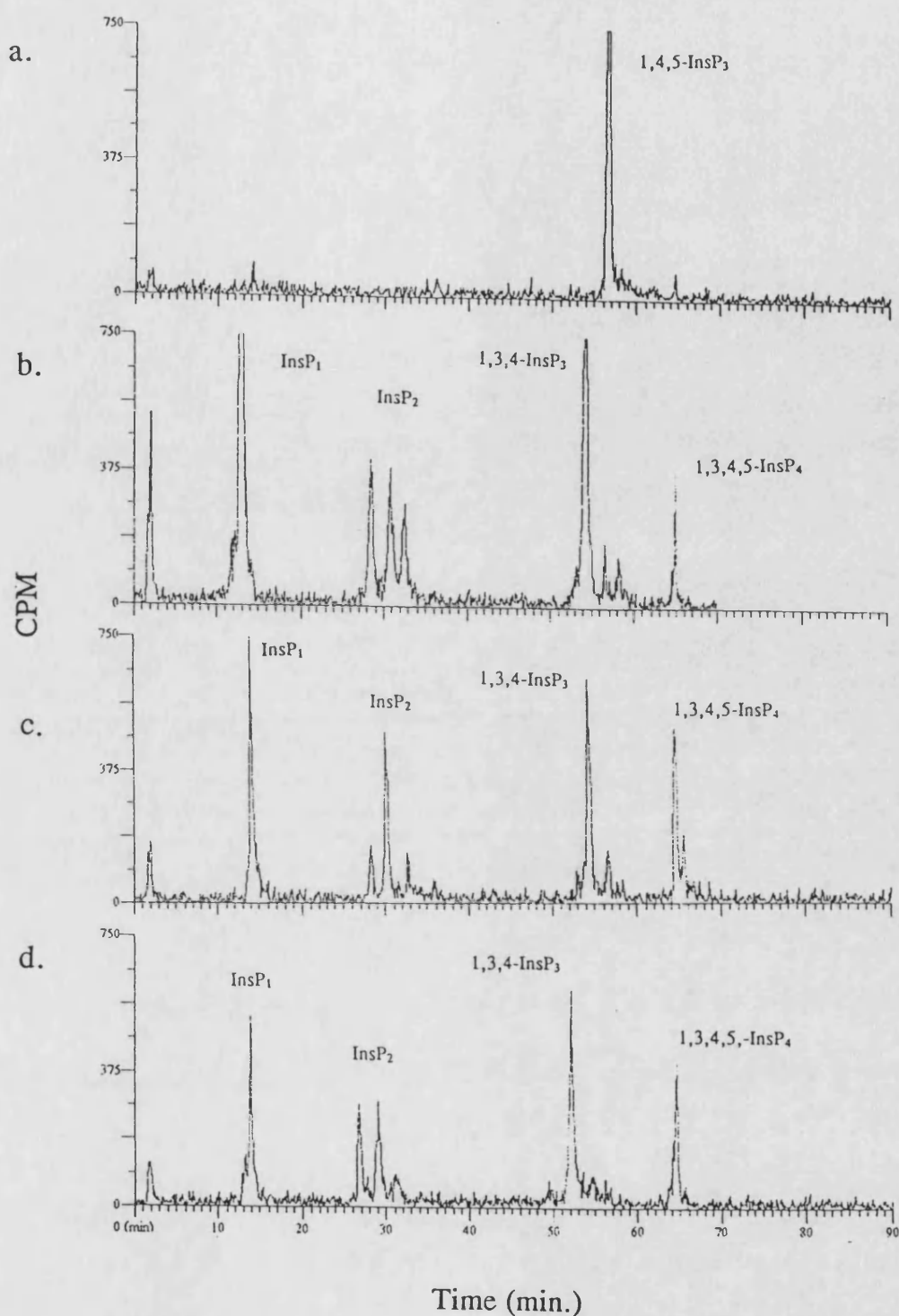


Figure 38. HPLC separation of $[^3\text{H}]\text{InsP}_3$ metabolites at 10 minutes in the presence of $3\mu\text{M}$ L- $\text{Ins}(1,4,5)\text{PS}_3$ and $\text{Ins}(1,3,5)\text{PS}_3$. Platelets were incubated with analogue for 5 minutes and challenged with $[^3\text{H}]\text{InsP}_3$, see methods. (a) $[^3\text{H}]\text{InsP}_3$ standard; (b) control InsP_3 metabolism ; (c) L- $\text{Ins}(1,4,5)\text{PS}_3$; (d) $\text{Ins}(1,3,5)\text{PS}_3$. Traces are representative of three separate experiments.

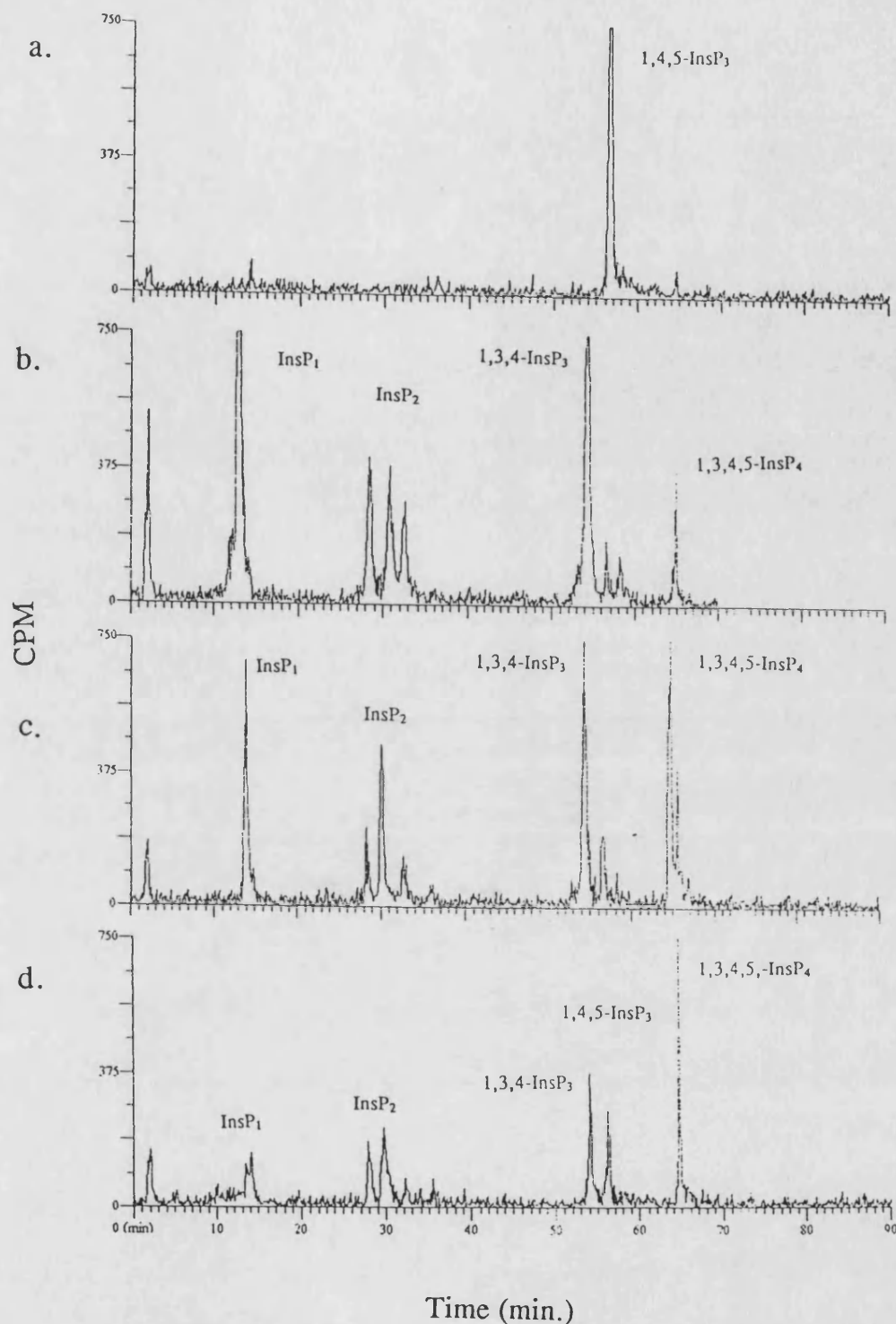


Figure 39. HPLC separation of $[^3\text{H}]\text{InsP}_3$ metabolites at 10 minutes in the presence of $10\mu\text{M}$ L-Ins(1,4,5) PS_3 and Ins(1,3,5) PS_3 . Platelets were incubated with analogue for 5 minutes and challenged with $[^3\text{H}]\text{InsP}_3$, see methods. (a) $[^3\text{H}]\text{InsP}_3$ standard; (b) control InsP_3 metabolism ; (c) L-Ins(1,4,5) PS_3 ; (d) Ins(1,3,5) PS_3 . Traces are representative of three separate experiments.

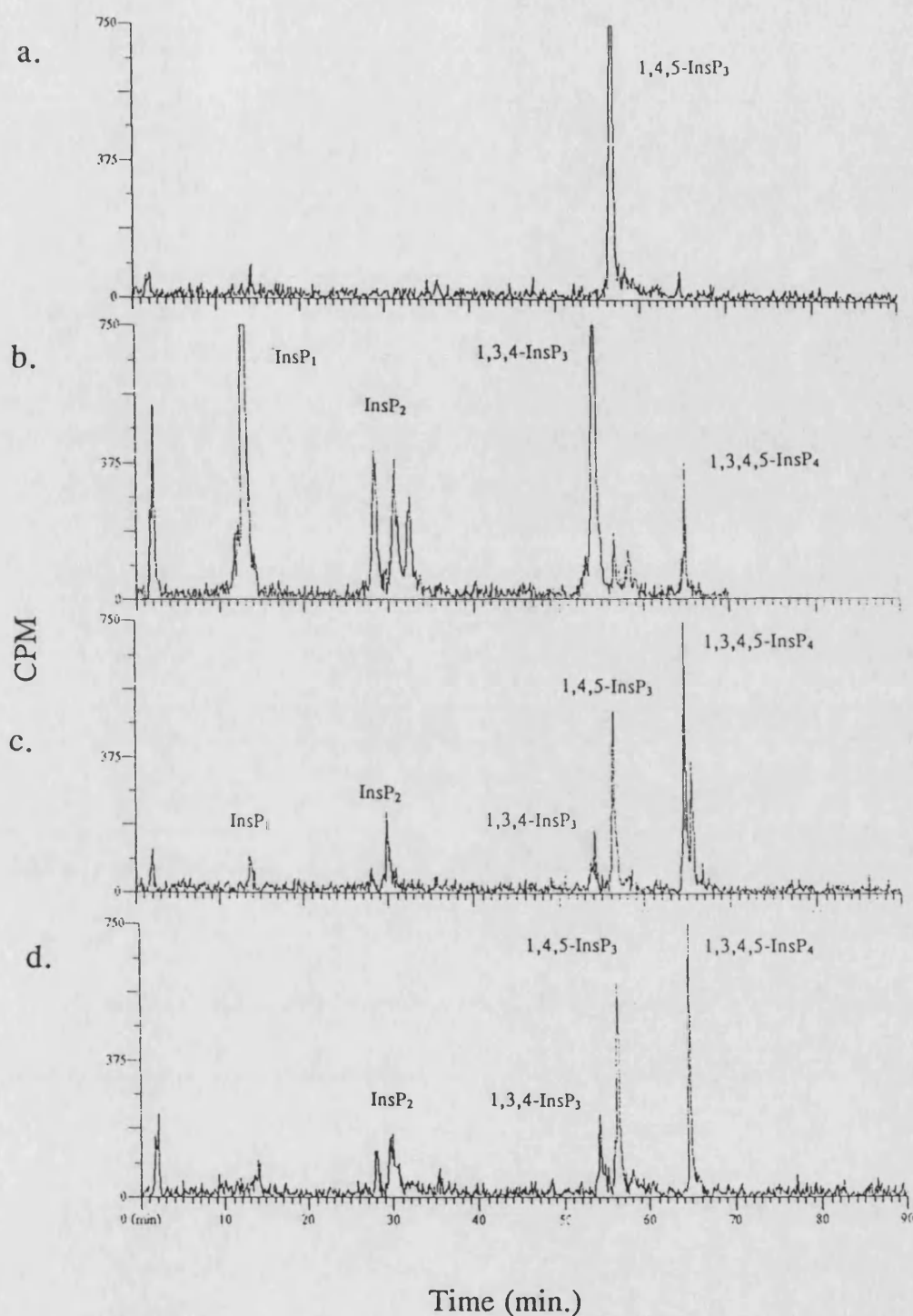


Figure 40. HPLC separation of $[^3\text{H}]\text{InsP}_3$ metabolites at 10 minutes in the presence of $30\mu\text{M}$ L-Ins(1,4,5) PS_3 and Ins(1,3,5) PS_3 . Platelets were incubated with analogue for 5 minutes and challenged with $[^3\text{H}]\text{InsP}_3$, see methods. (a) $[^3\text{H}]\text{InsP}_3$ standard; (b) control InsP_3 metabolism; (c) L-Ins(1,4,5) PS_3 ; (d) Ins(1,3,5) PS_3 . Traces are representative of three separate experiments.

enzyme. L-Ins(1,4,5)PS₃ also causes a dose dependent reduction in the amount of Ins(1,3,4,5)P₄ produced (compared to control) via the 3-kinase enzyme, but causes an inhibition of Ins(1,3,4)P₃ production (from Ins(1,3,4,5)P₄) compared to control. With L-Ins(1,4,5)PS₃ both levels of Ins(1,4)P₂ and InsP(1,3,4)P₃ are reduced, and from this a clear picture emerges, where the inhibition of the 5-phosphatase enzyme is observed. Thus L-Ins(1,4,5)PS₃ is acting as a 5-phosphatase inhibitor.

However regarding the effect of L-Ins(1,4,5)PS₃ on the 3-kinase enzyme, a definite effect is not apparent. Although an inhibition of Ins(1,3,4,5)P₄ production is observed, the levels are above that of the control, with the highest dose of 30μM L-Ins(1,3,5)PS₃ reducing levels to control levels.

Ins(1,3,5)PS₃ causes a dose dependent inhibition of InsP₃ metabolism, figure 42, with 30μM bringing levels of InsP₃ to the same levels of the control. It also produces a reduction in the amount of Ins(1,4)P₂ produced (compared to control), thus, inhibiting the 5-phosphatase enzyme. Ins(1,3,5)PS₃ causes an elevation in Ins(1,3,4,5)P₄ levels (above control), and a reduction in the amount of Ins(1,3,4)P₃ produced (compared to control), 30μM Ins(1,3,5)PS₃ causes levels of Ins(1,3,4)P₃ to be reduced to the same level as control .

3.2.3 Metabolism of [³²P]-Ins(1,3,4,5)P₄.

3.2.3.1 [³²P] incorporation into Ins(1,3,4,5)P₄.

In order to investigate the effect of the two enzyme inhibitors L-Ins(1,4,5)PS₃ and Ins(1,3,5)PS₃ on the 3-kinase enzyme specifically, [³²P]-ATP loaded platelets were used. After 10 minutes of incubation of the permeabilised platelets with [³²P]-ATP, no

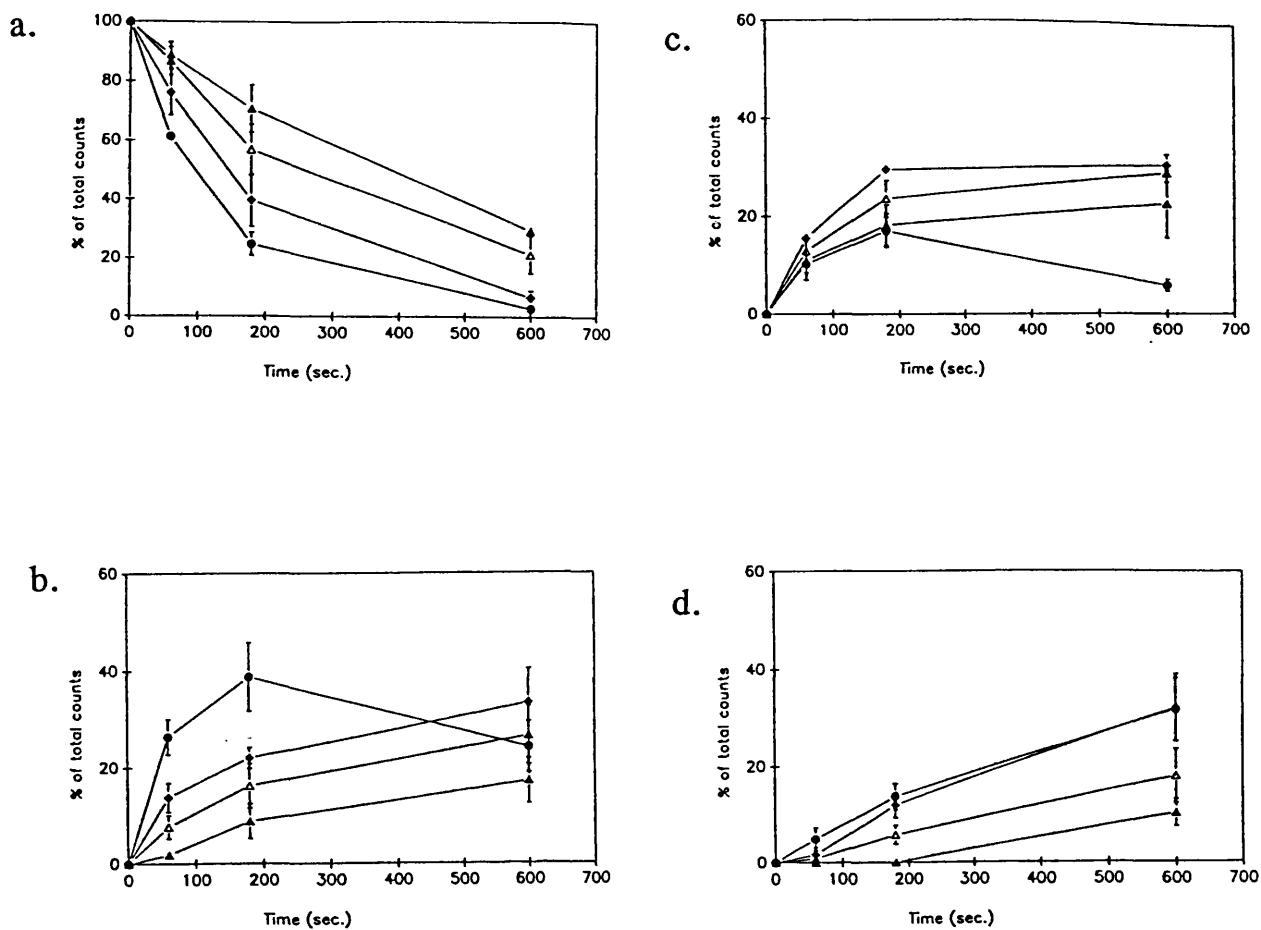


Figure 41. The effect of vehicle (●) or L-Ins(1,4,5)PS₃, 3 μM (◐), 10 μM (Δ) and 30 μM (▲) on (a) $[^3\text{H}]\text{InsP}_3$; (b) InsP_2 accumulation; (c) $\text{Ins}(1,3,4,5)\text{P}_4$ accumulation and (d) $\text{Ins}(1,3,4)\text{P}_3$ accumulation. Each point is mean \pm SE of three determinations.

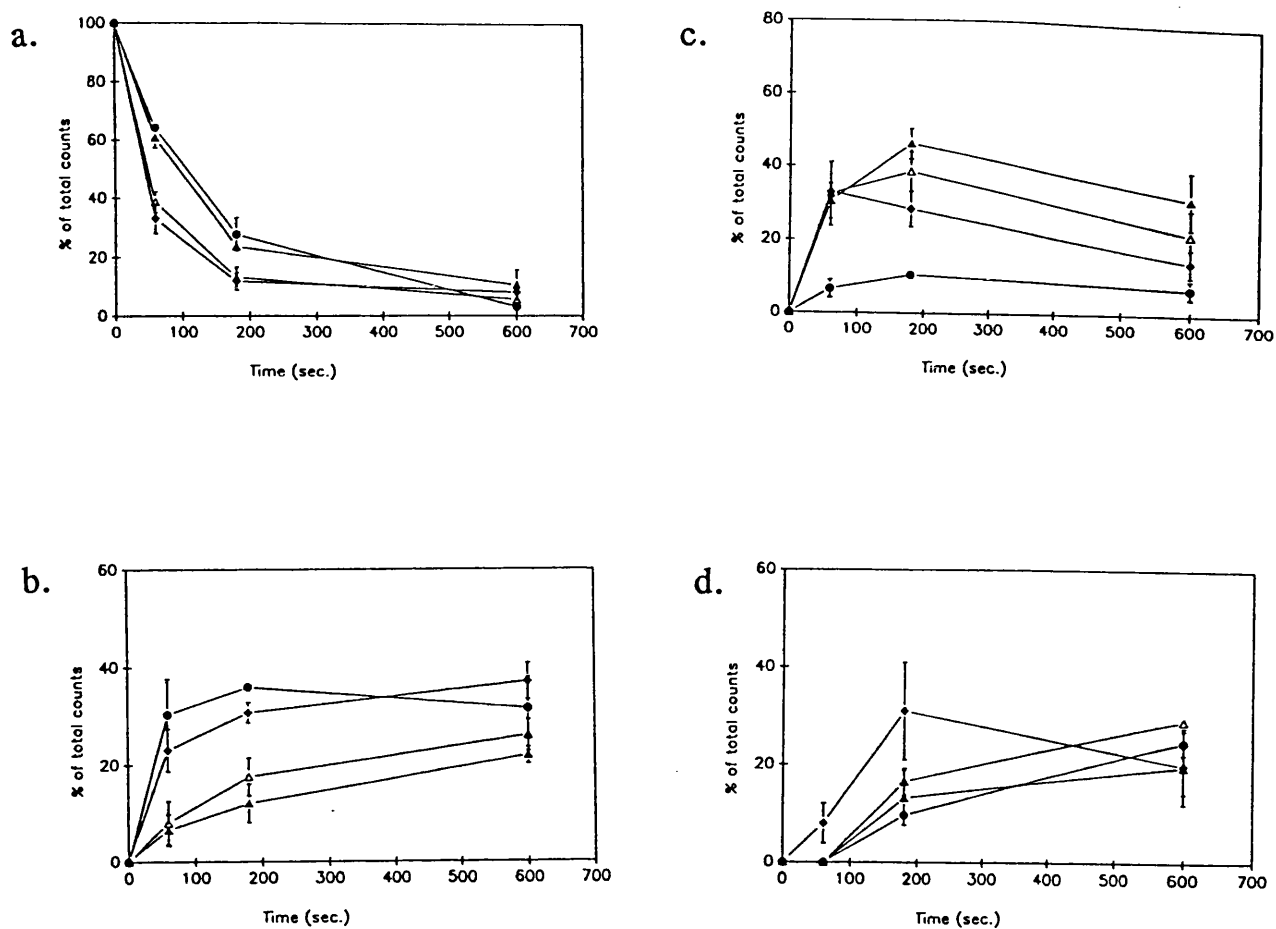


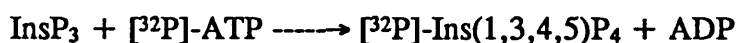
Figure 42. The effect of vehicle (●) or $\text{Ins}(1,3,5)\text{PS}_3$ 3 μM (◆), 10 μM (Δ) and 30 μM (▲) on (a) $[^3\text{H}]\text{InsP}_3$; (b) InsP_2 accumulation; (c) $\text{Ins}(1,3,4,5)\text{P}_4$ accumulation and (d) $\text{Ins}(1,3,4)\text{P}_3$ accumulation. Each point is mean \pm SE of three determinations.

sign of [^{32}P]-Ins(1,3,4,5) P_4 was detected. The traces obtained showed a clutter of undefinable peaks, representing [^{32}P]-ATP, between the retention times of $t = 0$ - 60 minutes. Ins P_3 appears in our system at 55- 57 minutes and Ins(1,3,4,5) P_4 appears at 62- 65 minutes. These areas were covered by the messy [^{32}P]-ATP peaks and it was not possible to establish the peak for [^{32}P]-Ins(1,3,4,5) P_4 from amongst them. In addition, because of the large amounts of [^{32}P]-ATP needed to carry out this experiment, it was not possible to reduce [^{32}P]-ATP which would in turn help resolve the peaks of interest. Furthermore, it was possible that the amount of intrinsic Ins P_3 available may not be enough to be detected after phosphorylation by [^{32}P]-ATP and to this end exogenous $30\mu\text{M}$ Ins P_3 was added at the same time of [^{32}P]-ATP was added, again [^{32}P]-Ins(1,3,4,5) P_3 was not detected.

3.2.3.2 The effect of inositol phosphate analogues on [^{32}P]-incorporation into Ins(1,3,4,5) P_3 .

In order to push metabolism via the 3-kinase pathway and increase the possibility of [^{32}P]-ATP induced phosphorylation of Ins P_3 , 5 minute pre-incubations of the permeabilised platelet with $30\mu\text{M}$ of the known 5-phosphatase inhibitors, L-Ins(1,4,5) PS_3 or $30\mu\text{M}$ Ins(1,3,5) PS_3 (see scheme) was investigated.

3-kinase



Scheme 1. [^{32}P]-incorporation into Ins(1,3,4,5) P_4 upon Ins P_3 phosphorylation.

Upon phosphate sample analysis on the HPLC, a peak for [^{32}P]-Ins(1,3,4,5) P_4 was not present, indicating that [^{32}P]-ATP phosphorylation of Ins P_3 had not

occurred via the 3-kinase enzyme and that [^{32}P]-incorporation into InsP_3 to produce [^{32}P]- $\text{Ins}(1,3,4,5)\text{P}_4$ had not occurred.

3.3 Platelet functional responses. Dense granule release induced by InsP_3 in permeabilised platelets.

3.3.1 InsP_3 induced Dense granule release in permeabilised platelets.

3.3.1.1 Saponin permeabilisation of platelets suspended in ICB.

Upon saponin permeabilisation of platelets resuspended in ICB with $[\text{Ca}^{2+}]_i$ 100- 200nM (with 5 $\mu\text{g}/\text{ml}$, 10 $\mu\text{g}/\text{ml}$ and 20 $\mu\text{g}/\text{ml}$ saponin), the amount of [^{14}C]5-HT loss upon permeabilisation was found to be dose dependent and time dependent, with the low concentration of 5 $\mu\text{g}/\text{ml}$ saponin causing no degranulation upon stimulation with 1mM Ca^{2+} , thus having no permeabilisation effect, 10 $\mu\text{g}/\text{ml}$ saponin showing 20% 5-HT release at 10 minute permeabilisation upon Ca^{2+} treatment and 20 $\mu\text{g}/\text{ml}$ showing considerable degranulation with 1mM Ca^{2+} at 5 minutes and above. The platelets do not degranulate with saponin treatment unless the highest concentration is used, 20 $\mu\text{g}/\text{ml}$, for 10 minutes (see figure 43). Hence 20 $\mu\text{g}/\text{ml}$ saponin causes sufficient permeabilisation at the longer time incubations of 5 and 10 minutes. However, at 10 minutes, substantial loss of 5-HT occurs with just vehicle treatment, therefore 5 minute incubations were used for any subsequent experiments.

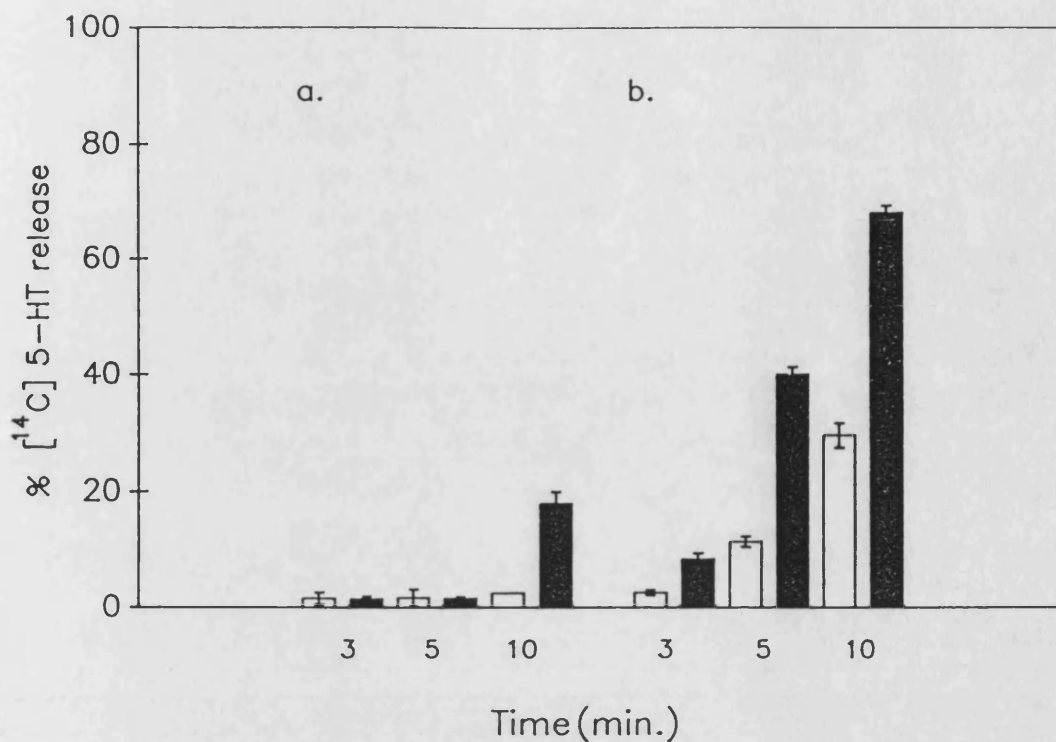


Figure 43. The effect of saponin permeabilisation, 10 $\mu\text{g/ml}$ (a) and 20 $\mu\text{g/ml}$ (b) on vehicle- (open bars) and on 1mM Ca^{2+} - (closed bars) induced $[^{14}\text{C}]$ 5-HT release. Platelets permeabilised with saponin 10 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$ for 3, 5 and 10 minutes, challenged with vehicle or 1mM Ca^{2+} for 3 min. and $[^{14}\text{C}]$ 5-HT release determined. Each point is the mean \pm SE of 3 determinations.

3.3.1.2 The effect of a dose range of Ca^{2+} on 20 $\mu\text{g}/\text{ml}$ saponin permeabilised platelets (5 minute incubations).

In order to establish that the above response to Ca^{2+} is a real response and not an anomaly a dose response effect to Ca^{2+} was investigated.

A dose dependent rise in [^{14}C]5-HT was observed (see figure 44) upon challenging platelets permeabilised with 20 $\mu\text{g}/\text{ml}$ saponin for 5 minutes with a dose range of Ca^{2+} , 300nM- 100 μM .

3.3.1.3 The effect of a dose range of EGTA on 20 $\mu\text{g}/\text{ml}$ saponin permeabilised platelets (5 minute incubations).

In order to ascertain the correlation between dense granule release and EGTA, [^{14}C]5-HT release, by a dose range of Ca^{2+} , was determined in 20 $\mu\text{g}/\text{ml}$ saponin permeabilised platelets in the presence of 30 μM or 3 μM EGTA.

In the presence of both EGTA doses, Ca^{2+} produced a dose dependent increase in degranulation, and this was in turn dependent on the amount of EGTA present. The lower EGTA dose producing a smaller chelation effect on the Ca^{2+} added, thus producing overall higher amounts of [^{14}C]5-HT release and 30 μM EGTA producing a greater chelation effect, with the dose ranges of Ca^{2+} releasing less [^{14}C]5-HT, see figure 45.

3.3.1.4 The effect of a dose of InsP_3 on saponin permeabilised platelets.

In platelets permeabilised with 10 $\mu\text{g}/\text{ml}$ and 20 $\mu\text{g}/\text{ml}$ saponin for 3, 5 and 10 minutes, 10 μM InsP_3 produced a [^{14}C]5-HT release above vehicle levels and greater release occurred with 20 $\mu\text{g}/\text{ml}$ saponin compared to 10 $\mu\text{g}/\text{ml}$ saponin, see figure 46. However, a large enough window did not exist between

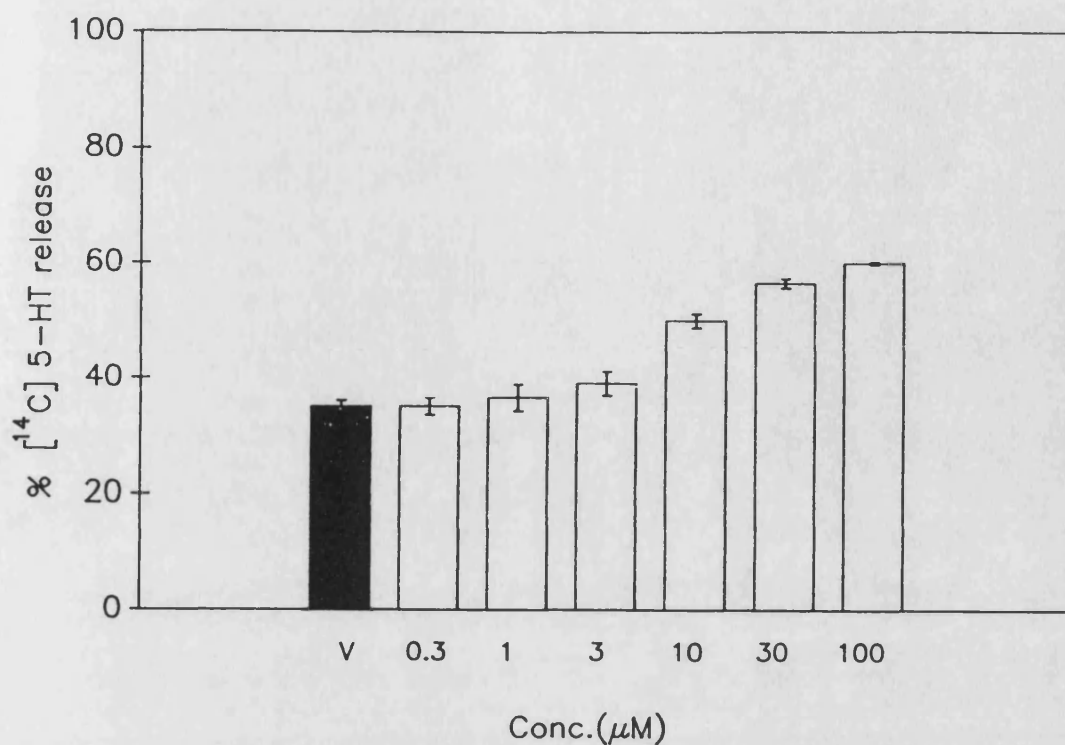


Figure 44. The effect of a dose range of Ca^{2+} on $[^{14}\text{C}]$ 5-HT release in saponin permeabilised platelets. Platelets permeabilised with $20\mu\text{g/ml}$ saponin, challenged with Ca^{2+} (300nM - $100\mu\text{M}$) (open bars) or vehicle (closed bars) and $[^{14}\text{C}]$ 5-HT release determined. Each point is the mean \pm SE of 3 determinations.

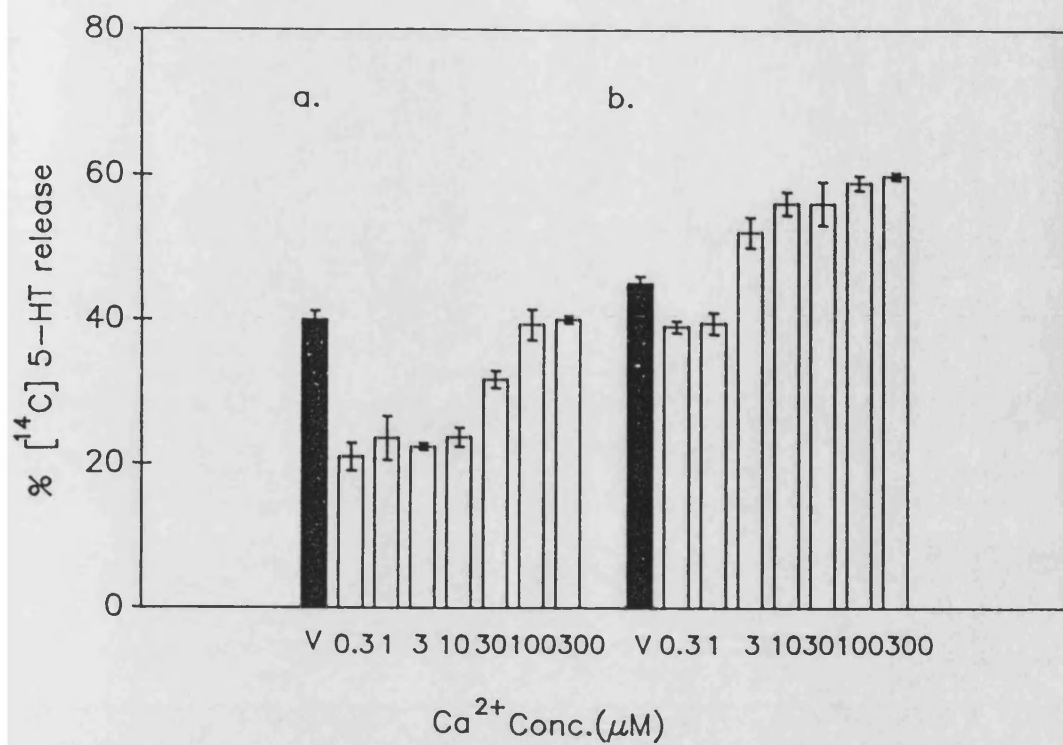


Figure 45. The effect of (a) 30 μM and (b) 3 μM EGTA on Ca²⁺- (open bars) or vehicle- (closed bars) induced [¹⁴C]5-HT release. Platelets permeabilised with saponin 20 μg/ml were treated with EGTA and then challenged with a dose range of Ca²⁺ (100 nM- 100 μM). Each point is the mean ± SE of 3 determinations.

the vehicle control and treated preparations to carry out any dose dependent studies with InsP_3 or analogues and this method was rejected.

3.3.1.5 Saponin permeabilisation of platelets suspended in high K^+ buffer.

$[^{14}\text{C}]5\text{-HT}$ released upon 1mM Ca^{2+} in platelets treated with different saponin concentrations ($5\mu\text{g/ml}$, $10\mu\text{g/ml}$, $15\mu\text{g/ml}$ and $20\mu\text{g/ml}$) was found to be dose dependent with $5\mu\text{g/ml}$ and $10\mu\text{g/ml}$ saponin causing very little dense granule release, $20\mu\text{g/ml}$ saponin causing extensive degranulation in the presence of vehicle only but $15\mu\text{g/ml}$ saponin at 5 minutes producing 12% release with vehicle and 35% release upon challenging with 1mM Ca^{2+} . Thus, $15\mu\text{g/ml}$ saponin concentration was used for the subsequent studies.

3.3.1.6 The effect of a dose range of EGTA on $15\mu\text{g/ml}$ saponin permeabilised platelets (3 minute incubations).

Upon treating platelets with a dose range of EGTA in the presence or absence of $10\mu\text{M InsP}_3$ (3 minute incubations), it was observed that at high EGTA concentrations, both vehicle and $10\mu\text{M InsP}_3$ treated platelets release comparable amounts of $[^{14}\text{C}]5\text{-HT}$, however as the $[\text{EGTA}]$ decreases, and reaches $20\mu\text{M}$, a small difference in release appears and this increases with decreasing $[\text{EGTA}]$, giving rise to a window of 15 % at $3\mu\text{M EGTA}$, see figure 47.

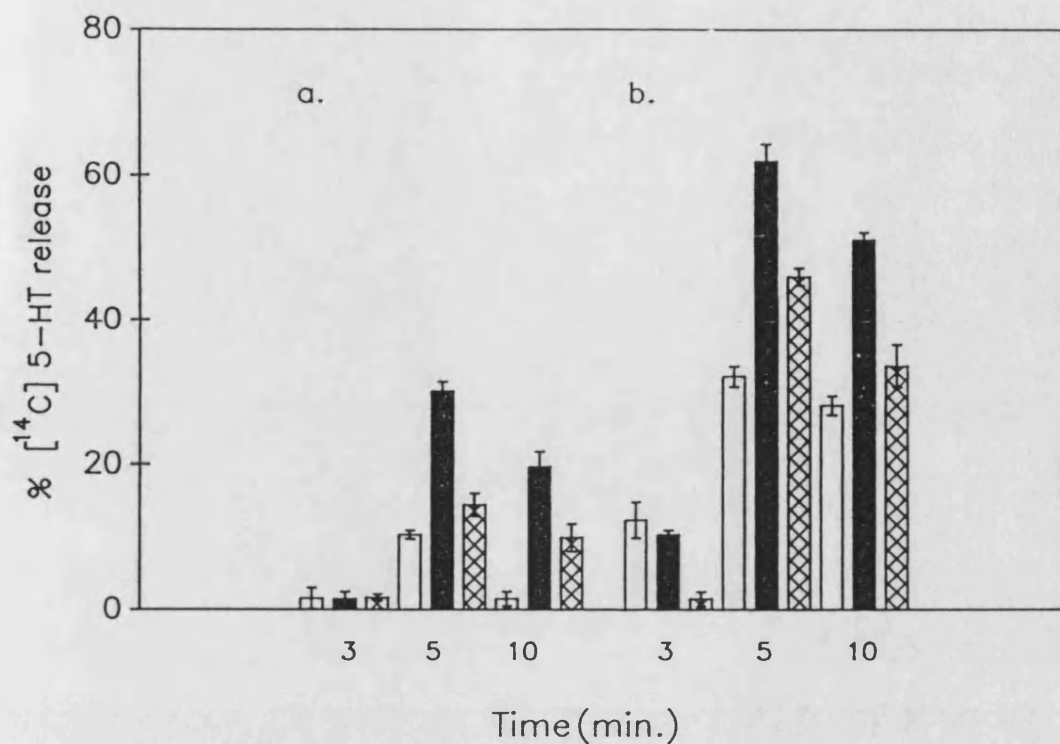


Figure 46. The use of different saponin concentrations, (a) 10μg/ml and (b) 20μg/ml in permeabilising platelets. Platelets were permeabilised with saponin for 3, 5 and 10 minutes then treated with vehicle (open bars); 1mM Ca²⁺ (closed bars) and 10μM InsP₃ (crosshatch) and [¹⁴C]5-HT release was determined. Each point is the mean ± SE of 3 determinations.

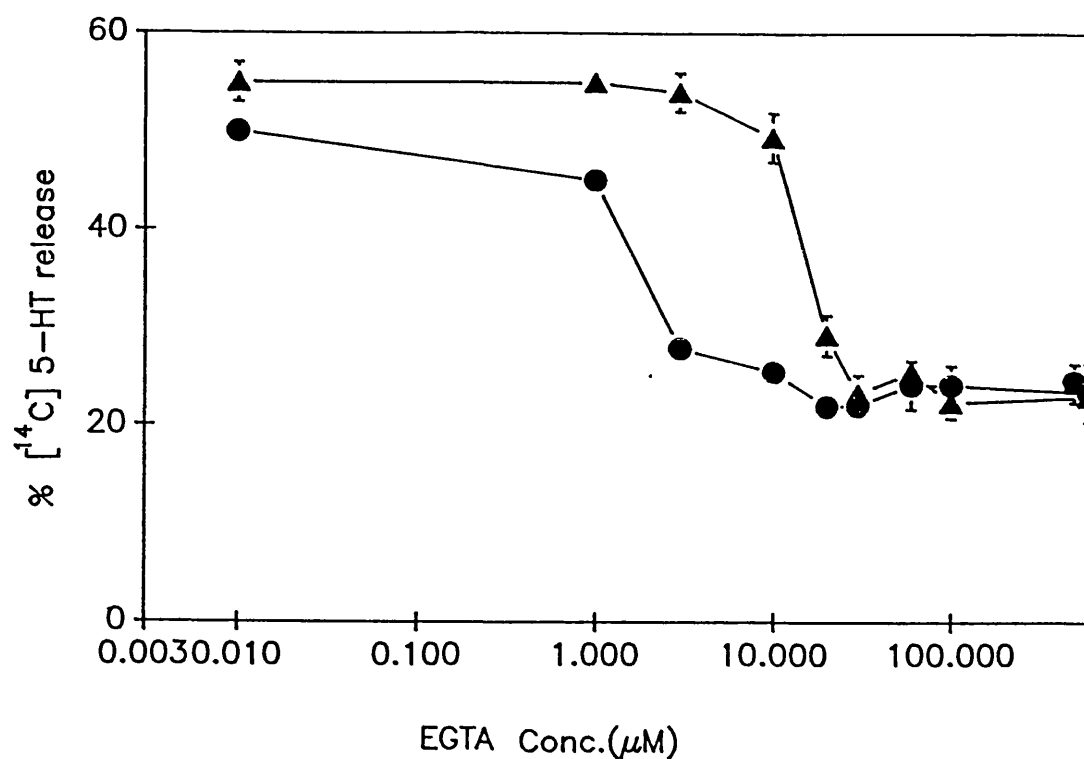


Figure 47. The effect of 3 minute incubations of 10μM InsP₃ on [¹⁴C]5-HT loaded permeabilised platelets in the presence of a dose range of EGTA. Platelets were loaded with [¹⁴C]5-HT, permeabilised with 20μg/ml saponin, treated with a dose range of EGTA and vehicle (●), or 10μM InsP₃ (▲), and [¹⁴C]5-HT release was determined. Each point is the mean ± SE of 3 determinations.

3.3.1.7 The effect of a dose range of InsP₃ on 15µg/ml saponin permeabilised platelets (3 minute incubations) in the presence of 30 or 10µM EGTA.

Platelets stimulated with a dose range of InsP₃ (300nM- 300µM) for 3 minutes in the presence of 30µM or 10µM EGTA did not produce a dose dependent increase in [¹⁴C]5-HT as expected. This could be on account of the [EGTA] being too high, and although a separation between InsP₃ treated and untreated platelets dense granule release was observed above, this may have been missed by choosing such a small range of [EGTA].

3.3.1.8 The effect of increasing contact time between platelets and agonist.

The longer incubation times when permeabilised platelets treated with a dose range of EGTA (1- 300µM) in the presence and absence of 10µM InsP₃ (5 and 7 minutes) had no effect on the magnitude of the window of separation between treated and control preparations (see figure 48), in fact it appears to have an opposite effect with only 10% separation occurring at 20µM EGTA.

Thus due to a small window between treated and untreated preparations, dense granule release can not be used to assess platelet function.

3.3.1.9 Investigation of electroporation of platelets suspended in ICB.

[¹⁴C]5-HT release, upon challenging platelets exposed to 2.2, 2.5, 3.5 and 5 kV at 5 and 10 cycle repetitions to varying concentrations of Ca²⁺ was a measure of permeability. At 2.2, 2.5 and 3.5 kV 5 and 10 cycles, [¹⁴C]5-HT release was not detected. At 5 kV both with 5 and 10 cycles, Ca²⁺ caused a dose dependent [¹⁴C]5-HT release indicating that the platelets were permeabilised, see figure 49.

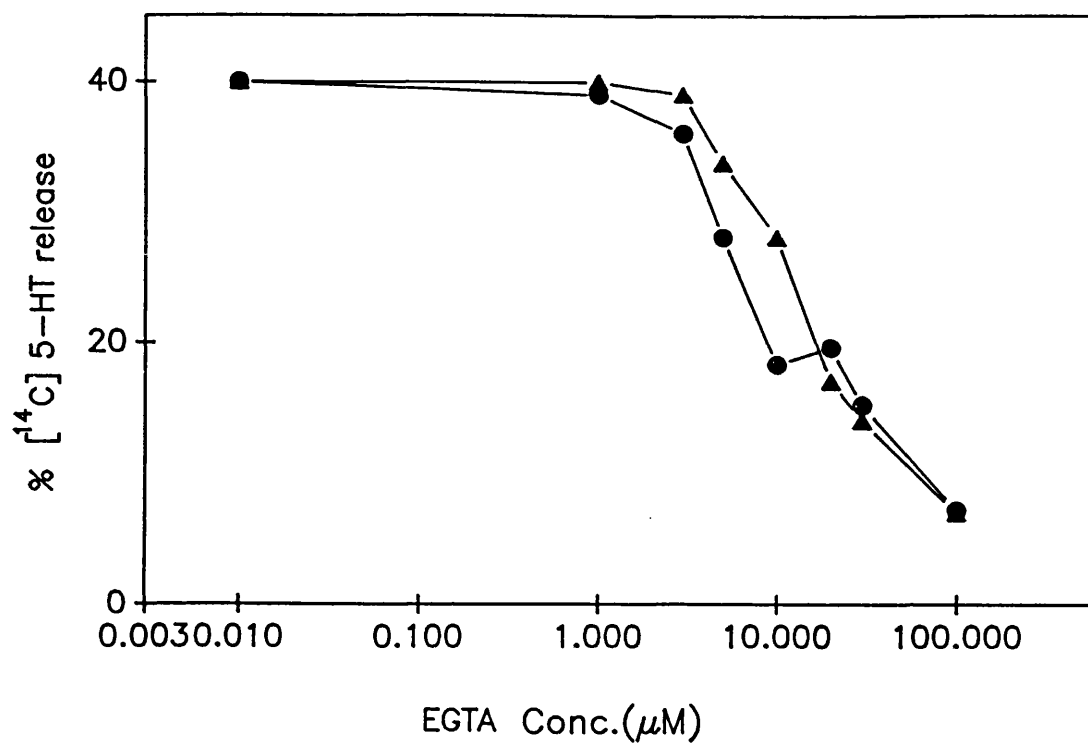


Figure 48. The effect of 5 minute incubations of 10μM InsP₃ on [14C]5-HT loaded permeabilised platelets in the presence of a dose range of EGTA. Platelets were loaded with [14C]5-HT, permeabilised with 20μg/ml saponin, treated with a dose range of EGTA and vehicle, (●), or 10μM InsP₃ (▲), and [14C]5-HT release was determined. Each point is the mean ± SE of 3 determinations.

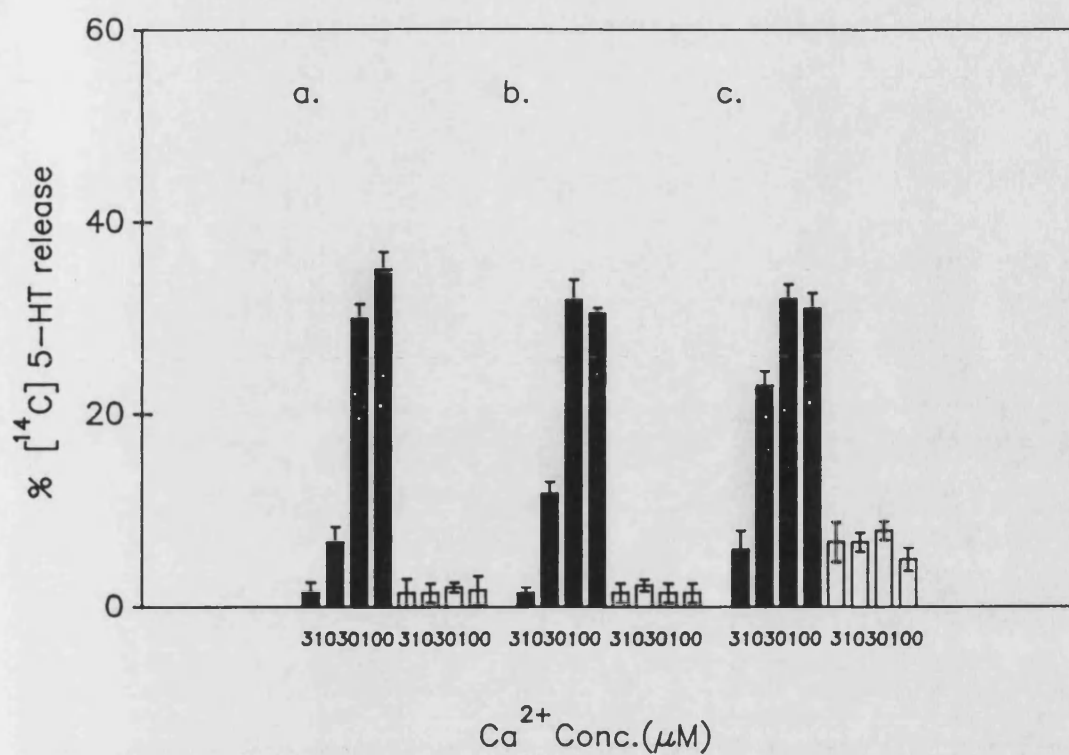


Figure 49. Ca²⁺-induced [¹⁴C]5-HT release in electroporated platelets at t=0 after poration (closed bars) and t=60 min. after poration (open bars) using (a) 5; (b) 10 and (c) 15 cycles at 5kV. [¹⁴C]5-HT release. Each point is the mean \pm SE of 3 determinations.

3.3.1.10 Investigation of resealing of electroporated platelets suspended in ICB.

Platelets porated with 5, 10 and 15 cycles of 5kV were challenged with Ca^{2+} (300nM- 1 μ M) immediately after poration (t=0) and after 30 minutes (t=30) and one hour incubations at 37°C (t=60).

Platelets were responsive to Ca^{2+} at t=0 minutes releasing [^{14}C]5-HT, but not after 30 minutes at 37°C, t=30 minutes, nor at t=60 minutes at 37°, see figure 49, indicating that after poration the platelets were permeable to Ca^{2+} ions and after 30 and 60 minutes at 37°C they were not, which raises the possibility of the platelets having resealed.

3.3.1.11 Investigation of the function of resealed electroporated platelets suspended in ICB.

Platelets electroporated with 10 cycles of 5kV did not respond to PAF (10nM- 300nM), see figure 50. In addition, after incubations at 37°C for 30 minutes and 1 hour these permeabilised platelets also did not respond to PAF and [^{14}C]5-HT release could not be detected in the presence or absence of 1mM Ca^{2+} .

3.3.1.12 Investigation of the function of resealed electroporated platelets suspended in high K^{+} buffer.

Platelets resuspended in high K^{+} buffer and electroporated with 5, 10 and 15 cycles of 5kV did not respond to PAF (10nM- 300nM). In addition, after incubations at 37°C for 30 minutes and 1 hour, PAF did not evoke a response and [^{14}C]5-HT release could not be detected.

Thus although apparent resealing takes place in so far as the platelets

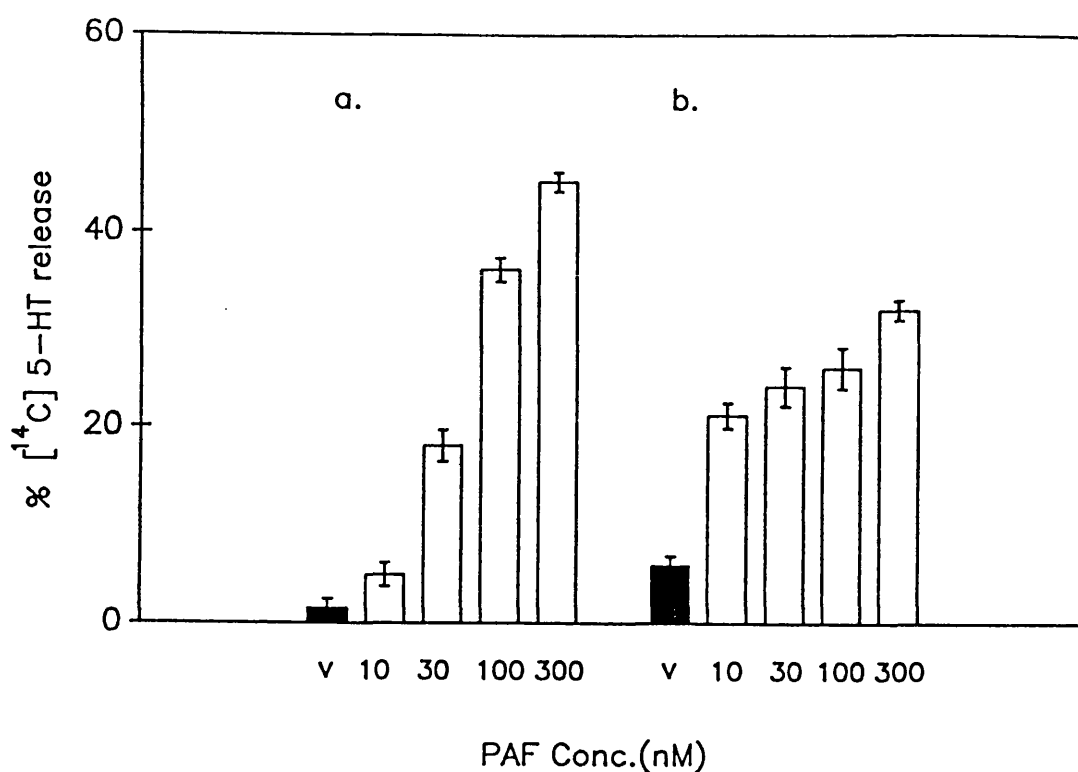


Figure 50. PAF-induced [^{14}C]5-HT release in (a) intact and (b) electroporated platelets. Platelets loaded with [^{14}C]5-HT were stimulated with (a) PAF (10-300nM) or (b) electroporated 10 cycles at 5kV and then challenged with PAF (10-300nM), [^{14}C]5-HT release is then determined. Each point is the mean \pm SE of 3 determinations.

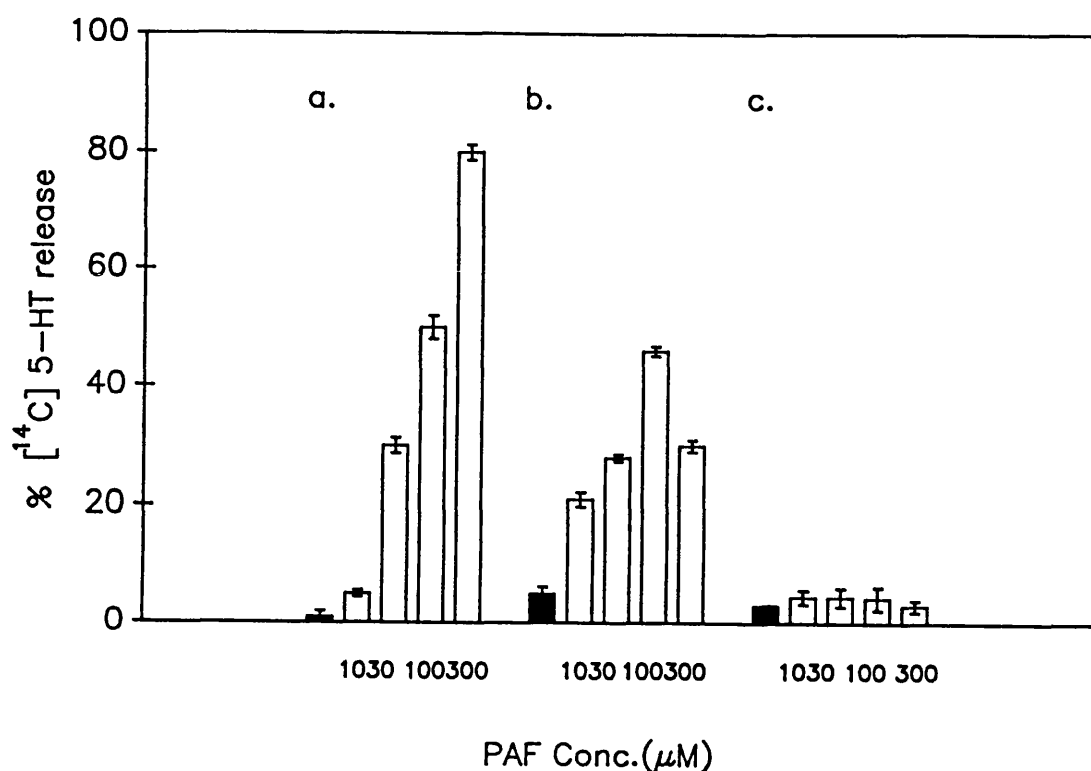


Figure 51. To assess permeabilisation and resealing of electroporated cells. Platelets loaded with [¹⁴C]5-HT and (a) intact platelets challenged with PAF (10- 300nM) (open bars) or vehicle (closed bars), (b) electroporated platelets (10 cycles at 5kV) challenged with PAF (10- 300nM) at t=0 after poration and (c) electroporated platelets (10 cycles at 5kV) challenged with PAF (10- 300nM) at t=60 after poration and [¹⁴C]5-HT release determined. Each point is the mean \pm SE of 3 determinations.

not responding to millimolar Ca^{2+} after incubations at 37°C in contrast to the responses to Ca^{2+} observed immediately after permeabilisation. However, the preparation is not responsive to PAF after 30- 60 minutes incubations at 37°C , indicating a breakdown in the signalling system. Thus with electroporation, platelets appear to permeabilise and possibly reseal but with the loss of platelet functional responses to extracellular agonists such as PAF, see figure 51.

3.4 Investigating the effects of the Ca^{2+} -influx inhibitor SKF 96365 on platelet function.

Ca^{2+} entry is thought to play an important role in Ca^{2+} elevation following receptor activation. SKF 96365 is an inhibitor of receptor-mediated calcium entry and has been used in this study to investigate the role played by extracellular Ca^{2+} in platelet activation.

3.4.1 The effect of SKF 96365 on Ca^{2+} release by PAF in intact Fura-loaded platelets.

Platelets loaded with the fluorescent indicator Fura-2, and challenged with PAF (3- 300nM) produced a dose dependent elevation in $[\text{Ca}^{2+}]$ with 3nM PAF producing a $1\mu\text{M}$ rise in $[\text{Ca}^{2+}]_i$. This dose was subsequently used to probe the effect the influx inhibitor would have on Ca^{2+} elevation, figure 52.

90 second pre-incubations of the platelets with a dose range of SKF 96365 (1- $100\mu\text{M}$) and then challenged with 3nM PAF, this produced a dose dependent inhibition of a 3nM PAF response with an IC_{50} of $21.1 \pm 1.1\mu\text{M}$ ($n=3$, mean \pm SE) and a reduction in Ca^{2+} elevation with 90% inhibition being achieved at $100\mu\text{M}$ SKF 96365, see figure 52.

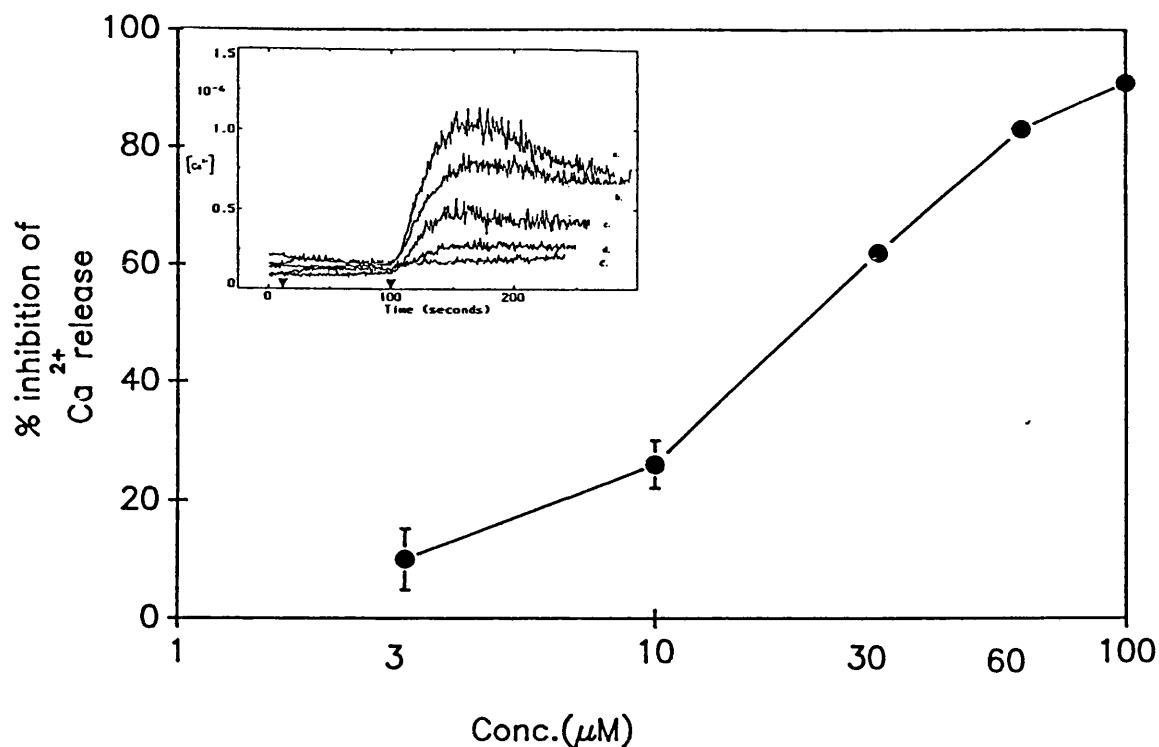


Figure 52. Inhibition of PAF-induced elevation of cytosolic calcium concentration by SKF 96365. Fura-2 AM loaded platelets were incubated with SKF 96365 for 90 seconds prior to stimulation with 3nM PAF. Each point is the mean \pm SE of 3 determinations. Inset shows representative traces of the dose dependent inhibition of 3nM PAF-induced Ca^{2+} -elevation by a: vehicle, b: 3 μ M SKF 96365, c: 10 μ M SKF 96365, d: 30 μ M SKF 96365, e: 100 μ M SKF 96365

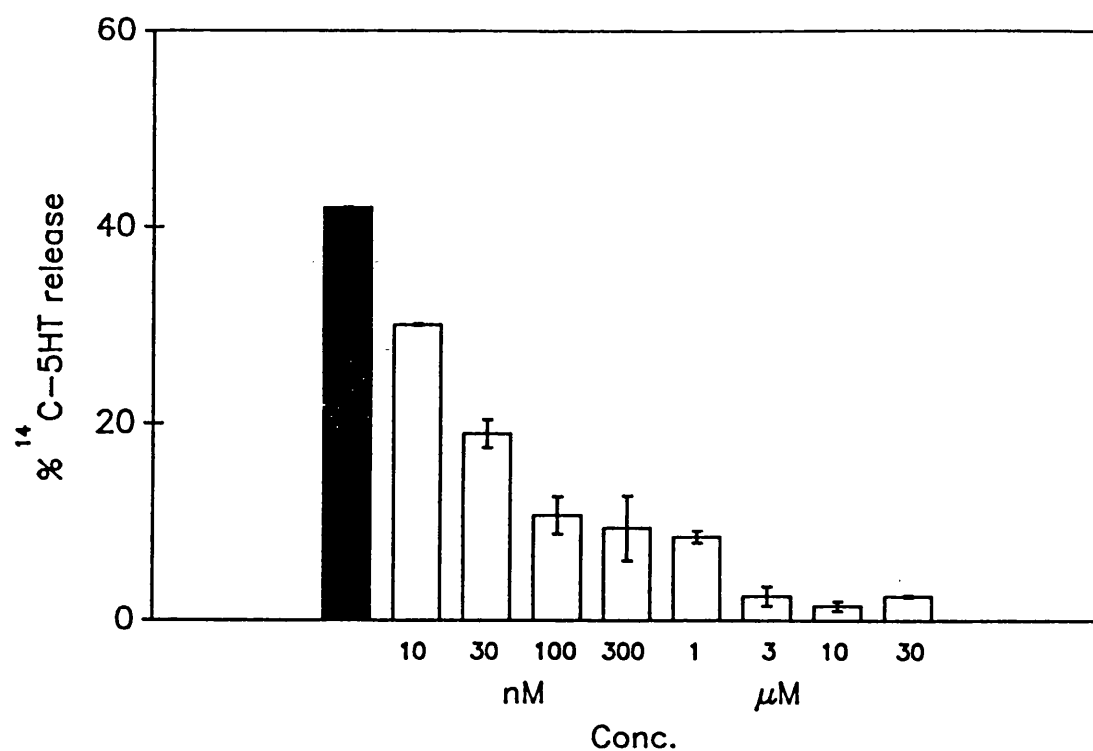


Figure 53. Inhibition of 3nM PAF-induced [¹⁴C]5-HT release by SKF 96365. [¹⁴C]5-HT loaded intact platelets were incubated with SKF 96365 for 90 seconds (open bars), or vehicle (closed bars), prior to activation by 3nM PAF. Each point is the mean \pm SE of 3 determinations.

3.4.2 The effect of SKF 96365 on dense granule release.

90 Second pre-incubation of platelets with SKF 96365 (1- 100 μ M) produced a dose dependent inhibition of dense granule release when platelets loaded with [¹⁴C]5-HT were challenged with 3nM PAF. Maximal inhibition was achieved with 10 μ M SKF 96365 with an IC₅₀ of 100nM \pm 20nM (n=3, mean \pm SE), see figure 53.

3.4.3 The effect of SKF 96365 on InsP₃ production.

90 Second pre-incubations of platelets with a dose range of SKF 96365 (1- 100 μ M) produced a dose dependent inhibition of InsP₃ production when stimulated with 300nM PAF, having an IC₅₀ of 24.3 \pm 3.2 μ M (n=3, mean \pm SE), see figure 54. Levels of InsP₃ rise from 4 \pm 2.7 pmol basal to 9.1 \pm 2.9 pmol InsP₃/10⁹ platelets upon maximal stimulation.

3.4.4 Studies on permeabilised platelets.

3.4.4.1 ⁴⁵Ca release induced by SKF 96365.

SKF 96365 (1- 100 μ M) challenged permeabilised platelets (20°C) had no capacity to mobilise Ca²⁺ from InsP₃-sensitive stores (see figure 55), in ⁴⁵Ca-loaded permeabilised platelets, in comparison to InsP₃ which releases a maximum of 75- 80% ⁴⁵Ca at 30 μ M, which has an EC₅₀ of 1.15 \pm 0.9 μ M

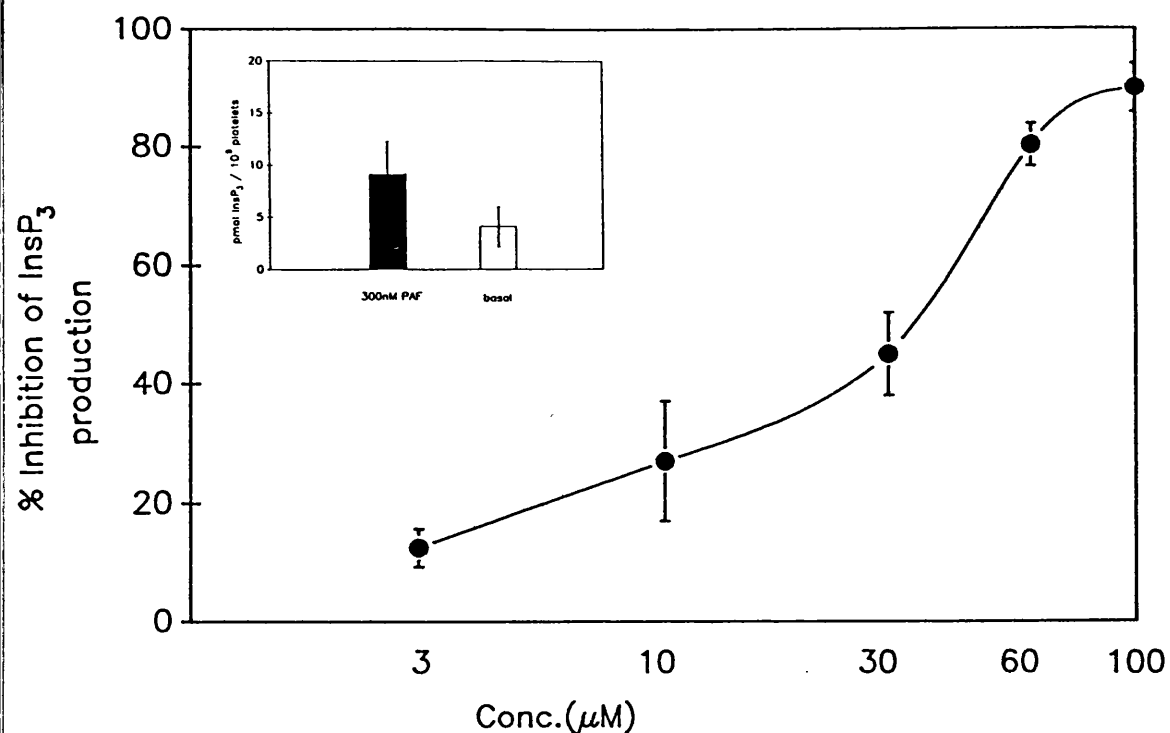


Figure 54. Inhibition of 300nM PAF-induced InsP₃ production by SKF 96365. Platelets were incubated with SKF 96365 for 90 seconds prior to activation by 300 nM PAF and InsP₃ production was determined. Inset shows InsP₃ levels at basal and maximum stimulation. All values have been obtained by subtracting the basal pre-PAF from those obtained after PAF-stimulation. Each point is the mean \pm SE of 3 determinations.

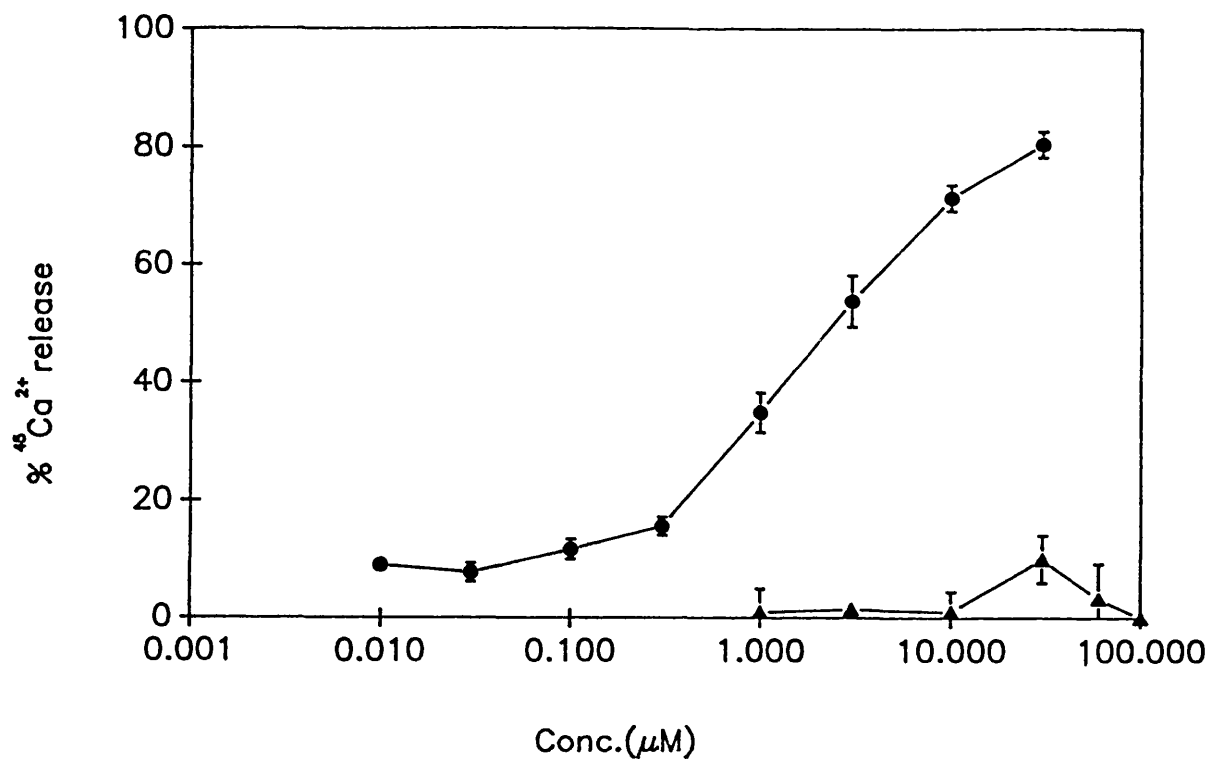


Figure 55. Dose response curves to InsP₃ (●) and SKF 96365 (▲) for their ability to release sequestered ⁴⁵Ca from saponin permeabilised platelets. Permeabilised platelets were loaded with ⁴⁵Ca, stimulated with a dose range of InsP₃ or SKF 96365 for 3 minutes at 20°C and ⁴⁵Ca release was determined by rapid filtration. Each point is the mean ± SE of 3 determinations.

3.4.4.2 The effect of SKF 96365 on InsP₃ and Ins(1,3,4,5)P₄-stimulated ⁴⁵Ca release.

SKF 96365 (1- 100 μ M) had no effect of both 3 μ M InsP₃ and 10 μ M Ins(1,3,4,5)P₄ ⁴⁵Ca-induced mobilisation at 20°C (see figure 56 and 57). 3 μ M InsP₃ releases 68 \pm 2% (n=3, mean \pm SE) of the sequestered ⁴⁵Ca, whereas 10 μ M Ins(1,3,4,5)P₃ release 28 \pm 3% (n=3, mean \pm SE) of the loaded ⁴⁵Ca (at 20°C), figure 58.

3.5 The use of crude platelet membranes for examining InsP₃-induced Ca²⁺ release.

3.5.1 ⁴⁵Ca release assay.

3.5.1.1 Optimal cell number and protein concentration for ⁴⁵Ca.

The amount of ⁴⁵Ca taken up into the membrane preparation was directly proportional to the protein concentration, with the greater protein concentration having a greater amount of ⁴⁵Ca associated with it.

Thus for subsequent experiments platelets were suspended to 10⁹ cell/ml and later freeze/thawed for membrane preparation, figure 59.

	Protein conc. mg/ml	% ⁴⁵ Ca uptake
Platelets /ml		
10 ⁸	0.1	22 \pm 3
2x10 ⁸	0.3	31 \pm 1.6
10 ⁹	0.95	38 \pm 2.1

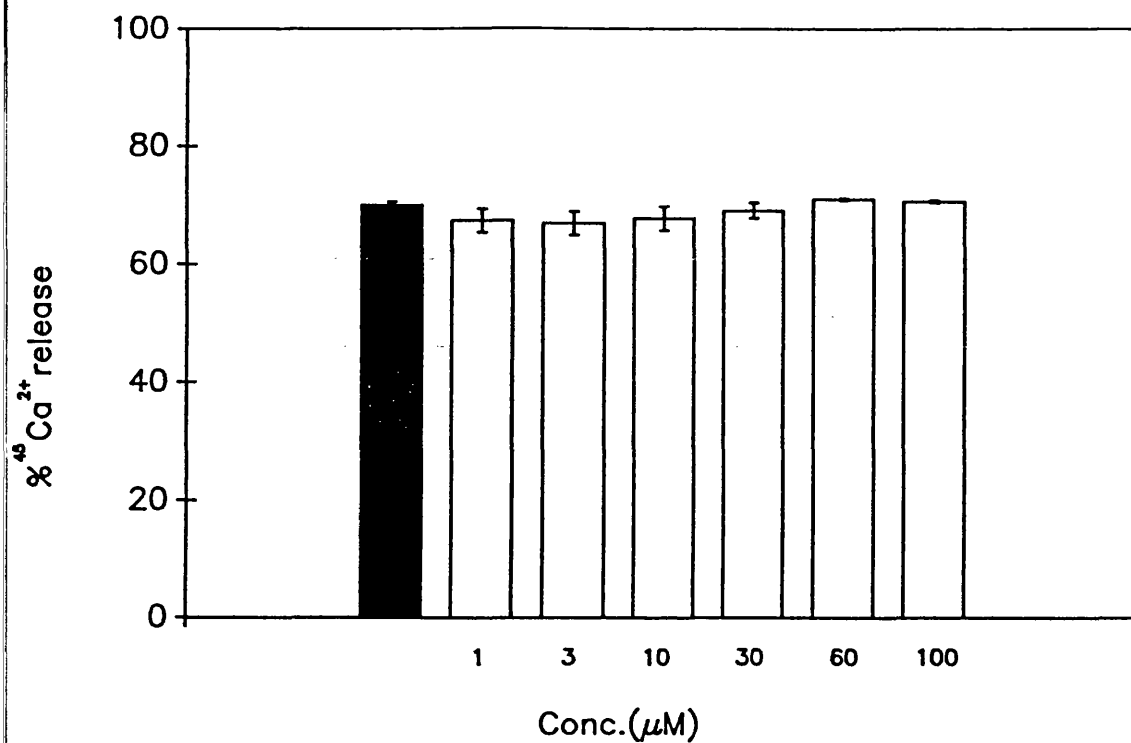


Figure 56. The effect of SKF 96365 on $3\mu\text{M}$ InsP_3 -induced ^{45}Ca -release. Saponin permeabilised platelets were incubated with vehicle (closed bars), or SKF 96365 (open bars) for 90 seconds prior to challenge with $3\mu\text{M}$ InsP_3 for 3 minutes at 20°C . ^{45}Ca release determined by rapid filtration. Each point is the mean \pm SE of 3 determinations.

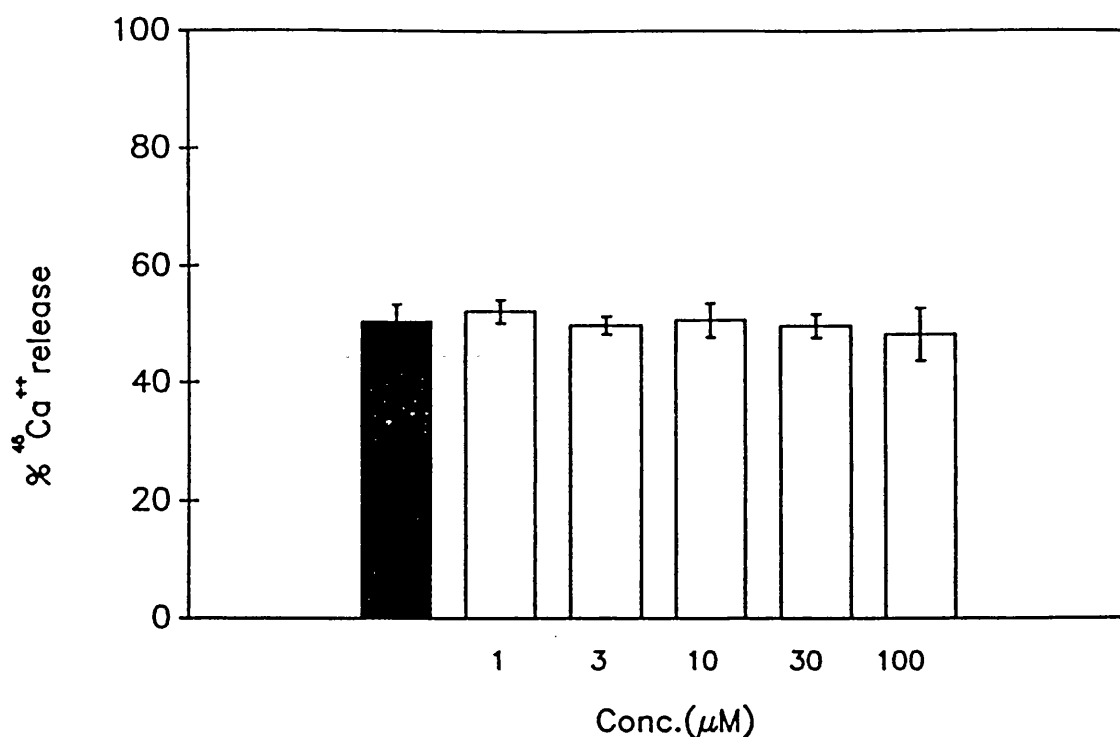


Figure 57. The effect of SKF 96365 on 3μM Ins(1,3,4,5)P₄-induced ⁴⁵Ca-release. Saponin permeabilised platelets were incubated with vehicle (closed bars), or SKF 96365 (open bars) for 90 seconds prior to challenge with 10μM Ins(1,3,4,5)P₄ for 3 minutes at 20°C. ⁴⁵Ca release determined by rapid filtration. Each point is the mean ± SE of 3 determinations.

3.5.1.2 Time course of ^{45}Ca uptake into crude platelet membranes.

^{45}Ca uptake was time dependent reaching a plateau after 30 minutes of incubation with ^{45}Ca . The plateau was maintained for over one hour which is sufficient to carry out a release experiment. The overall percentage of ^{45}Ca uptake into these membranes, in comparison to uptake into permeabilised platelets, was found to be considerably less, 29% in comparison to 45%, figure 60.

3.5.1.3 InsP₃ dose response curve in crude membrane preparations.

Crude platelet membranes prepared on three separate occasions, when challenged with InsP₃, produced three different dose/response relationships, see figure 60. In addition, the dose response curves are nominal in that the EC₅₀ are very high indicating little receptor activation or the presence of a low receptor concentration. This with the afore mentioned inconsistency in response suggests that the platelet membrane preparations on the different days are different with respect to InsP₃ receptor concentration. The reason for this is not known. Thus, crude membrane preparation can not be used to study InsP₃- and analogue-induced ^{45}Ca release.

3.5.1.4 The effect of heparin on InsP₃-induced ^{45}Ca release.

Heparin (300ng/ml- 100 μg /ml) caused a dose dependent inhibition in crude rabbit membranes and 100% inhibition was not achieved. However, comparable to the inconsistency of InsP₃-induced ^{45}Ca experienced above, heparin tested on membranes prepared on separate occasions produced different degrees of

inhibition (see figure 61). Thus, an IC_{50} value could only be obtained for each individual experiment and these could not be compared directly.

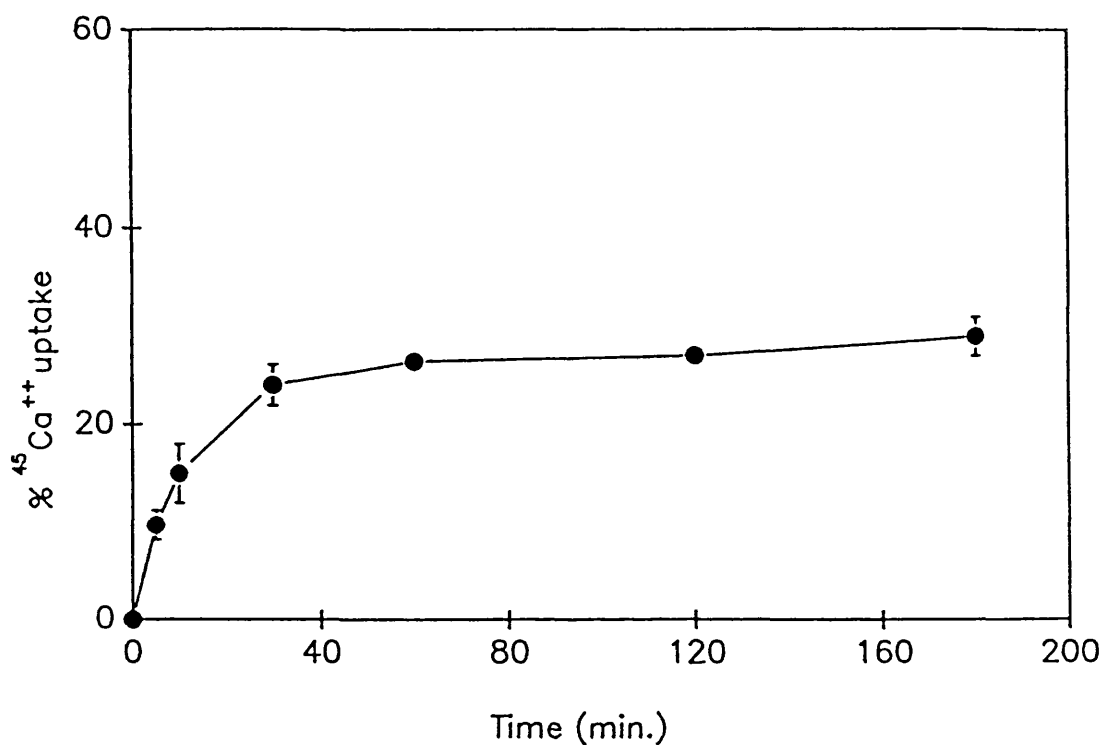


Figure 59. Time course of ^{45}Ca uptake into crude platelet membrane preparations. Crude platelet membranes were prepared and ^{45}Ca uptake at predetermined times was measured over a period of 2 hours. Each point is the mean \pm SE of 3 experiments.

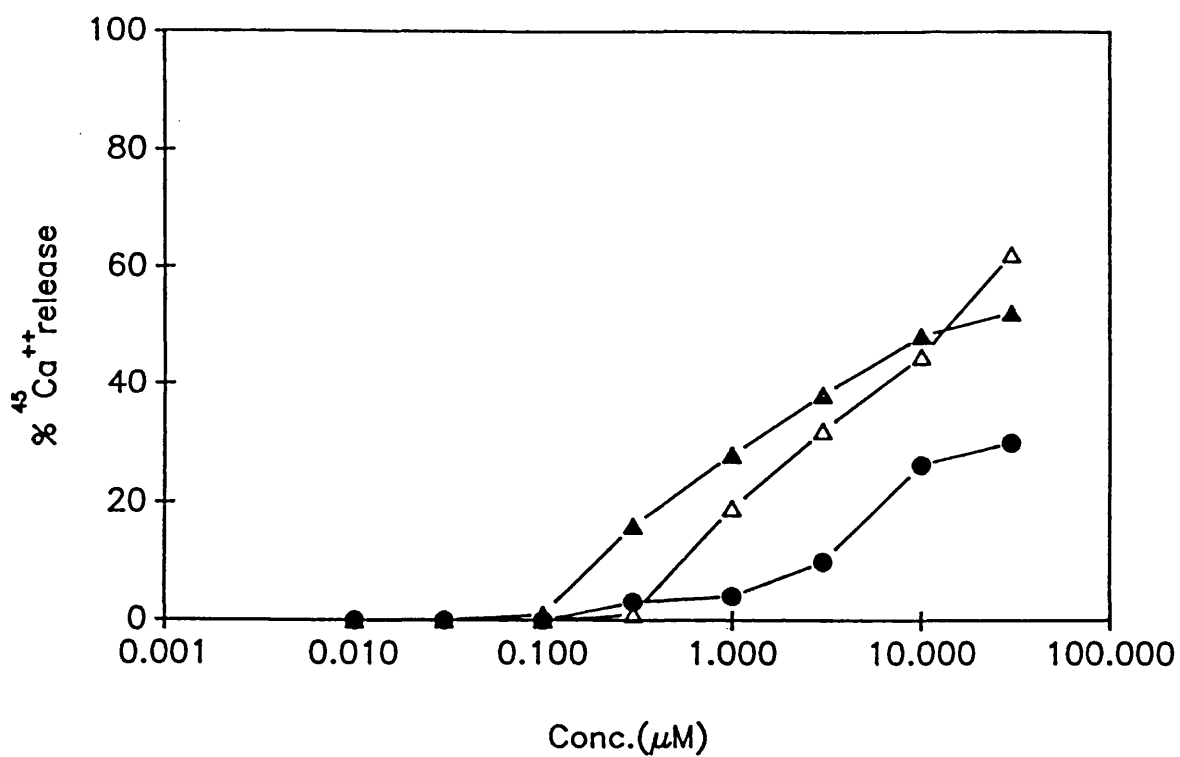


Figure 60. Dose response curves to InsP_3 of three different crude membrane preparations. Crude membranes were prepared on three separate days, loaded with ^{45}Ca and challenged with a dose range of InsP_3 for 3 minutes. This graph shows the variation in response to InsP_3 of the three separate preparations.

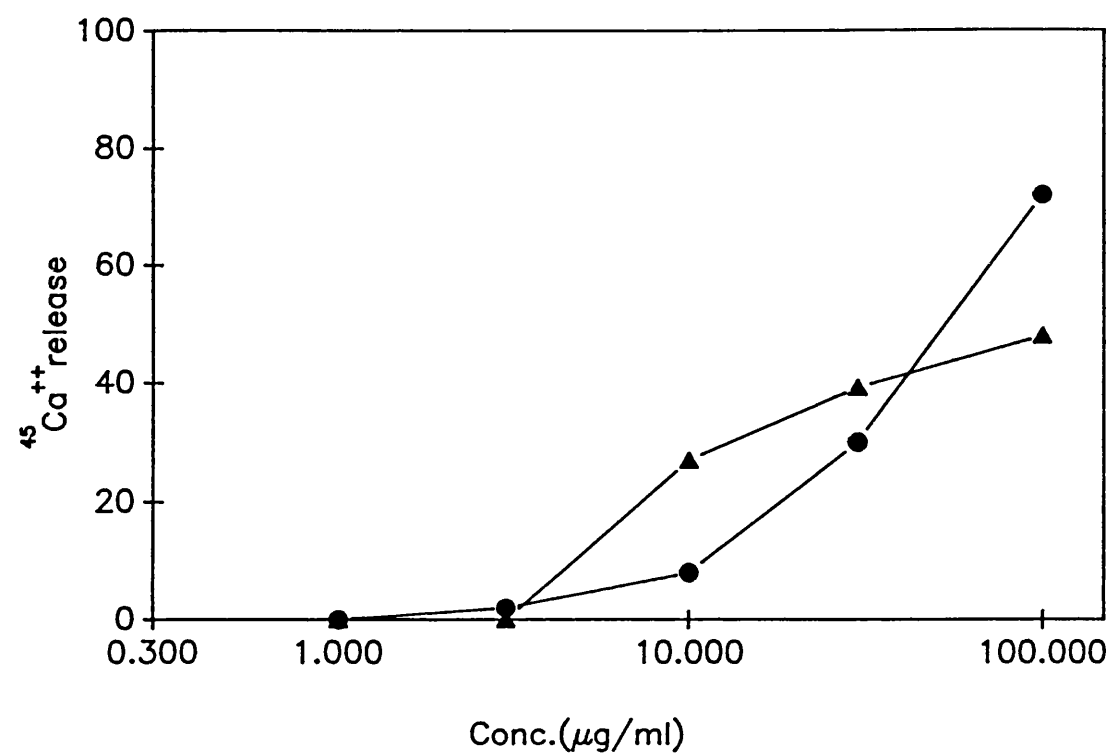


Figure 61. Inhibition of $3\mu\text{M}$ InsP_3 -induced ^{45}Ca -release in crude platelet membranes by heparin. Crude platelet membranes loaded with ^{45}Ca were pre-incubated with heparin ($1\mu\text{g/ml}$ - $100\mu\text{g/ml}$) for 3 minutes and then challenged with $3\mu\text{M}$ InsP_3 for 3 minutes at 20°C . Graphs are results of two membrane preparations and show a marked variation in response.

DISCUSSION

4.1 Structure/activity relationships: The requirements of the InsP₃ receptor for Ca²⁺ release.

4.1.1 Structural requirements for Ca²⁺ release.

Intact platelets stimulated with InsP₃ did not release Ca²⁺. This indicates that these cells did not become permeable upon incubation with InsP₃ in ICB, and that they need to be permeable in order for InsP₃ to elicit a response. Upon saponin treatment, platelets became sensitive to InsP₃.

The ability of InsP₃ to mobilise Ca²⁺ from intracellular stores was found to be stereospecific, as demonstrated by a comparison of D-InsP₃ and L-InsP₃. D-InsP₃ released 75- 85% of non-mitochondrial Ca²⁺ with an EC₅₀ of $1.14 \pm 0.2 \mu\text{M}$ (at 20°C), whereas L-InsP₃ was unable to mobilise Ca²⁺ (at all concentrations examined). This is consistent with previous reports (Strupish *et al.*, 1988) in which L-InsP₃ was found to be some 2000-fold weaker than D-InsP₃ and that it exhibited very low affinity for the InsP₃-binding site showing no partial agonism or antagonism at the InsP₃ receptor (Strupish *et al.*, 1988; Polokoff *et al* 1988)

4.1.2 Temperature dependence of InsP₃-induced Ca²⁺ release.

InsP₃-induced Ca²⁺-release was found to be very temperature dependent. The EC₅₀ at 4°C, 20°C and 37°C were $0.18 \pm 0.1 \mu\text{M}$, $1.14 \pm 0.2 \mu\text{M}$ and $\geq 10 \mu\text{M}$ respectively. Thus, approximately 50 fold more InsP₃ is required to release the same amount of ⁴⁵Ca at 37°C compared to 4°C.

The causes of this marked temperature dependence could include (a). a reduced binding affinity of ligand with receptor at 37°C, (b). temperature dependent loss

of intrinsic Ca^{2+} mobilisation, (c). a loss in the concentration of InsP_3 due to increased metabolism, (d). loss in activity of Ca^{2+} -channel at 37°C .

In our system, optimal ^{45}Ca release was observed at 4°C and the rank order for Ca^{2+} -release is $4^\circ\text{C} > 20^\circ\text{C} > 37^\circ\text{C}$. This is reflected by the rates of InsP_3 metabolism, see later. The order of efficiency of metabolism is $37^\circ > 20^\circ\text{C} > 4^\circ\text{C}$ and greater ^{45}Ca release at the lower temperatures is an indication of lower enzyme activity. Thus, at lower temperatures, a higher proportion of the exogenously applied InsP_3 is conserved. Because InsP_3 can reach a higher concentration at 4°C in the vicinity of the receptor, due to this reduced metabolism, it is capable of releasing a greater proportion of sequestered Ca^{2+} . However, InsP_3 remains equally efficacious.

Our data suggests a rapid loss of InsP_3 at 37°C due to metabolism. However, it is still possible that other factors such as a. and b. above are contributing to these differences in EC_{50} at 4°C , 20°C and 37°C . In order elucidate the role of metabolism in this apparent decrease in potency with increasing temperature, studies on metabolic-resistant analogues need to be carried out.

The initial curves for InsP_3 - and *scyllo*- $\text{Ins}(1,2,4,5)\text{P}_4$ -induced Ca^{2+} release at 20°C , indicate that the latter is apparently more potent than InsP_3 at mobilising Ca^{2+} . However, upon studying the Ca^{2+} release profiles of *scyllo*- $\text{Ins}(1,2,4,5)\text{P}_4$ at 4°C , 20°C and 37°C , it was established that *scyllo*- $\text{Ins}(1,2,4,5)\text{P}_4$ -induced Ca^{2+} release is also temperature dependent. The EC_{50} at 4°C , 20°C and 37°C are $0.1 \pm 0.05 \mu\text{M}$, $0.1 \pm 0.05 \mu\text{M}$ and $5.1 \pm 0.2 \mu\text{M}$ respectively. At 4°C , InsP_3 and *scyllo*- $\text{Ins}(1,2,4,5)\text{P}_4$ were equally potent in their release ability. At 20°C , InsP_3 appears to be 10 fold less potent than *scyllo*- $\text{Ins}(1,2,4,5)\text{P}_4$ and at 37°C , InsP_3 appears to be only 2-fold less potent.

Of interest is the finding that the EC_{50} for *scyllo*-Ins(1,2,4,5)P₄ at 4°C and 20°C are identical ($0.1 \pm 0.05 \mu\text{M}$). This may be due to metabolism, and *scyllo*-Ins(1,2,4,5)P₄ may be metabolised slowly at these two temperatures.

In addition, we can probably attribute the greater potency of *scyllo*-Ins(1,2,4,5)P₄ at 20°C, in comparison to InsP₃, to a metabolism effect, InsP₃ being metabolised at a faster rate than *scyllo*-Ins(1,2,4,5)P₄ at 20°C. This, presumably implies that *scyllo*-Ins(1,2,4,5)P₄ is a weaker substrate for the metabolic enzymes than InsP₃, or that it is even an inhibitor of these enzymes. The use of stable analogues of *scyllo*-Ins(1,2,4,5)P₄ may help elucidate the role of metabolism in this case. However, *scyllo*-Ins(1,2,4,5)PS₄, a stable phosphorothioate analogue of *scyllo*-Ins(1,2,4,5)PS₄, has been examined but found to be a partial agonist in this system and hence unable to define the role of metabolism here.

4.1.3 Phosphorothioate modification.

Phosphorothioate analogues are of considerable interest due to their resistance to intracellular phosphatases (Willcocks *et al.*, 1988) and their potential use in investigating metabolism. A number of analogues of InsP₃ were synthesized in order to design a phosphatase resistant analogue. Three InsP₃ phosphorothioate analogues were examined, Ins(1,4,5)PS₃, Ins(1)P(4,5)PS₂ and D/L-Ins(1)PS(4,5)P₂ all of which were found to be full agonists, releasing Ca²⁺ with similar potencies to InsP₃ with EC_{50} of $0.8 \pm 0.1 \mu\text{M}$, $0.85 \pm 0.1 \mu\text{M}$ and $0.65 \pm 0.1 \mu\text{M}$ respectively, at 20°C. All three compounds were thus as potent as InsP₃ in mobilising ⁴⁵Ca at 20°C. Further, it appears that complete substitution of all the phosphates with phosphorothioates, as with Ins(1,4,5)PS₃, does not affect potency when comparing with selective substitution at the vicinal 4,5-

bisphosphate, as in Ins(1)P(4,5)PS₂ and D/L-Ins(1)PS(4,5)P₂. Thus, the substitution of phosphates with phosphorothioates causes only a slight loss in potency at the platelet receptor. Various studies have reported that Ins(1,4,5)PS₃ is a persistent activator of InsP₃-receptor, being resistant to metabolism by the InsP₃ 5-phosphatase (Taylor *et al.*, 1989; Wojcikiewicz *et al.*, 1990; Safrany *et al.*, 1991a). The enantiomer of Ins(1,4,5)PS₃, L-Ins(1,4,5)PS₃, was found to be inactive. This is consistent with the stereoselectivity displayed by the InsP₃-receptor, as previously discussed, where L-InsP₃ was found to be inactive in comparison to D-InsP₃. In addition, this compound did not display any inhibitory effect on InsP₃-induced mobilisation. This is not a surprise, since L-Ins(1,4,5)PS₃ was designed as a non-Ca²⁺ mobilising 5-phosphatase inhibitor.

Inositol phosphorothioates are finding considerable applications in studies of the polyphosphoinositide signalling pathway, (Potter, 1990bc; Potter, 1993). Luckhoff and Clapham (1992) in their recent work give a direct demonstration of activation by Ins(1,3,4,5)P₄ of a Ca²⁺-channel in endothelial cells using phosphorothioate analogues.

In all cases where the phosphorothioate analogue, Ins(1,4,5)PS₃ has been used, its metabolic stability was crucial for these experiments. These compounds are proving to be valuable pharmacological and biochemical tools and will continue to help solve some of the problems encountered in PI signalling. For review of phosphorothioate analogues see (Potter, 1990ac; Potter, 1991; Potter, 1993).

4.1.4 Positional modifications.

The role of the three phosphates (-1, -4 and -5) and the three hydroxyls (2-, 3- and 6-) in Ca²⁺ mobilisation has been investigated in rabbit platelets. This was

achieved by the availability of various ring modified and phosphate modified analogues in order to understand the contribution and significance of these phosphates and hydroxyl groups to Ca^{2+} mobilisation.

4.1.4.1 Modification at the 1-position.

The introduction of a phosphorothioate at the 1-position is tolerated as depicted in D/L-Ins(1)PS(4,5)P₂. This compound is a potent agonist with an EC₅₀ of $0.65 \pm 0.1 \mu\text{M}$ at 20°C. It appears, then, that small changes in the nature of the group on the 1-position have little effect on Ca^{2+} -mobilising ability.

In addition, from the literature, a number of semisynthetic derivatives of InsP₃ substituted at the 1-position have been investigated (Henne *et al.*, 1988) and it was shown that the introduction of glyco- and glycerol-based large groups at this position does not produce a major loss in activity.

4.1.4.2 Modification at the 2-position.

Ins(2,2)F₂(1,4,5)P₃ has two fluorine atoms replacing its axial 2-OH and the equatorial 2-hydrogen. The axial 2-fluorine is expected to mimic the axial 2-OH group, both sterically and electronically. This analogue was found to be as potent as InsP₃ in mobilising Ca^{2+} in permeabilised platelets, with an EC₅₀ of $0.63 \pm 0.09 \mu\text{M}$ at 20°C, indicating that a major change to the 2-position in terms of functional group and ionic environment has little effect on receptor binding and mobilisation of Ca^{2+} . Thus, this reveals that the 2-position plays a very minor role in InsP₃'s action. These results are consistent with studies in SH-SY5Y neuroblastoma cells (Safrany *et al.*, 1990; Safrany *et al.*, 1992a) and the work of Hirata *et al* (1990; 1993).

Ins(1,2,4,5)P₄, a synthetic InsP₃ analogue, possesses an additional phosphate on the 2-position, as well as the phosphates on the 1-position and vicinal 4,5-position of InsP₃. This analogue was found to be a full agonist in permeabilised platelets with an EC₅₀ of 0.53 ± 0.04 μM at 20°C and is as potent as InsP₃. This supports the idea that the requirements for InsP₃ receptor binding are fulfilled by this analogue and represents the first observation that a charged anionic group at the 2-position does not affect activity to any great extent.

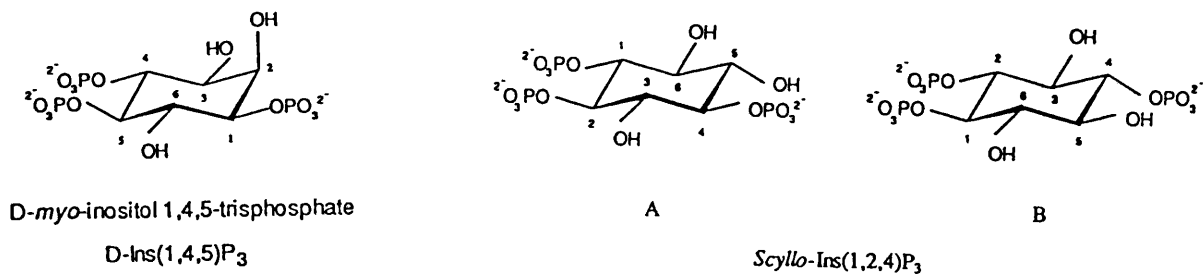


Figure 62. The structures of InsP₃ and the possible two binding modes of *Scyllo*-Ins(1,2,4)P₃.

InsP ₃	<i>scyllo</i> -Ins(1,2,4)P ₃	comparison of -OH
2 axial	5 equatorial	Different
3 equatorial	6 equatorial	Same
6 equatorial	5 equatorial	Same

Table 4. Comparison of the position and orientation of the hydroxyls of InsP₃ and *Scyllo*-Ins(1,2,4)P₃

It is not apparent immediately how this molecule may interact with the InsP₃ receptor. However, it is possible to envisage two modes of *scyllo*-Ins(1,2,4)P₃ binding to the InsP₃ receptor, see figure 62.

Scyllo-Ins(1,2,4)P₃ and InsP₃ are the same in terms of the regiochemistry of their phosphates, see figure 63, but InsP₃ possesses an axial 2-OH which is replaced by an equatorial 5-OH in *scyllo*-Ins(1,2,4)P₃, see table 4. However, the equatorial 3-OH of InsP₃ is matched by the equatorial 6-OH of *scyllo*-Ins(1,2,4)P₃ and the equatorial 6-OH of InsP₃ is mimicked by the equatorial 3-OH of *scyllo*-Ins(1,2,4)P₃. *Scyllo*-Ins(1,2,4)P₃ is a full agonist as potent as InsP₃ in releasing Ca²⁺ with an EC₅₀ of $0.58 \pm 0.07 \mu\text{M}$. This maintained activity despite 2-OH modification, from axial to equatorial, indicates that the 2-OH is not essential for Ca²⁺ mobilisation and receptor occupation.

The introduction of large groups at the axial 2-position is tolerated, leading to a slight loss in activity (Hirata *et al.*, 1990). The above data all indicate that the 2-position is relatively unimportant for receptor recognition and Ca²⁺ mobilisation in this study using permeabilised platelets.

4.1.4.3 Modification at the 3-position.

The role of the 3-OH of InsP₃ is of particular interest as it is the site of phosphorylation by InsP₃ 3-kinase, and its deletion would be of interest. However, 3-deoxy analogues were not available for evaluation in this study.

L-chiro-Ins(2,3,5)P₃ is modified at the 3-position, see figure 63, where the equatorial 3-OH of InsP₃ is replaced by an axial 1-OH of *L-chiro*-Ins(2,3,5)P₃ resulting in an inversion of stereochemistry.

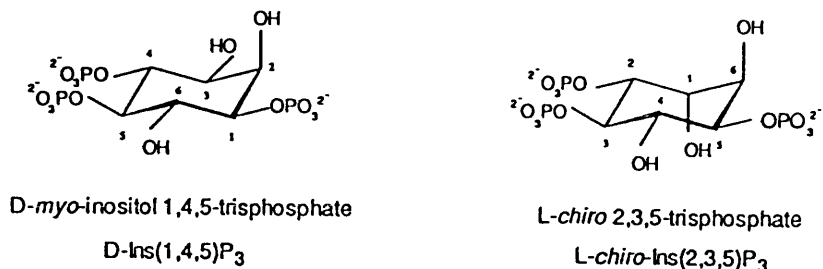


Figure 63. The structures of InsP₃ and L-*chiro*-Ins(2,3,5)P₃

InsP₃ L-*chiro*-Ins(2,3,5)P₃ comparison of -OH

2 axial	6 axial	same
3 equatorial	1 axial	Different
6 equatorial	4 equatorial	Same

Table 5. Comparison of the position and orientation of the hydroxyls of InsP₃ and L-*chiro*-Ins(2,3,5)P₃

This analogue relative to InsP₃, has the same pseudo D-1,4,5- phosphates as InsP₃ and the orientation of the 2- and 6-OH groups of InsP₃ are the same as those of L-*chiro*-Ins(2,3,5)P₃, see table 5. The 2- and 6-OH groups of InsP₃ are the same as the 6- and 4-OH groups of L-*chiro*-Ins(2,3,5)P₃ respectively. L-*chiro*-Ins(2,3,5)P₃ shows full agonist properties with an EC₅₀ of $1.9 \pm 0.1 \mu\text{M}$ at 20°C, and is only 1.7 -fold less potent than InsP₃ at 20°C. This indicates that the 3-OH, surprisingly plays a minor role in InsP₃ binding and Ca²⁺ release, especially when we consider that the action of the 3-kinase is at this position.

Safrany *et al* (1992b) and Liu *et al.*, (1992) used SH-SY5Y neuroblastoma cells to evaluate this analogue and it was found to be a full agonist with an EC₅₀ of

$1.4 \pm 0.3 \mu\text{M}$ whereas InsP_3 has an EC_{50} of $0.09 \mu\text{M}$, and this is in agreement with our findings.

The natural metabolite $\text{Ins}(1,3,4,5)\text{P}_4$ was found to be weak in mobilising Ca^{2+} in permeabilised platelets, having an EC_{50} of $\geq 10 \mu\text{M}$ at 20°C , thus being \approx 10-fold less potent than InsP_3 . A phosphorothioate analogue of $\text{Ins}(1,3,4,5)\text{P}_4$, $\text{Ins}(3)\text{PS}(1,4,5)\text{P}_3$, designed as an $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase inhibitor, was slightly less potent than the parent phosphate in mobilising Ca^{2+} , as seen in the dose response curves, with an EC_{50} of $\geq 10 \mu\text{M}$. $\text{Ins}(3)\text{PS}(1,4,5)\text{P}_3$ has the same phosphates as $\text{Ins}(1,3,4,5)\text{P}_4$ at the 1-, 4- and 5-positions and, although it possess a phosphorothioate at 3-position, it might be expected to be as potent as $\text{Ins}(1,3,4,5)\text{P}_4$. $\text{Ins}(3)\text{PS}(1,4,5)\text{P}_3$ was shown to be slightly less potent than $\text{Ins}(1,3,4,5)\text{P}_4$ at mobilising Ca^{2+} from the platelet InsP_3 receptor. This suggests that substitution of a phosphate with a phosphorothioate at the 3-position of $\text{Ins}(1,3,4,5)\text{P}_4$ does not have a significant effect on Ca^{2+} mobilisation activity.

$\text{Ins}(1,3,4,5)\text{P}_4$ was found to mobilise Ca^{2+} in other cell systems. In SH-SY5Y neuroblastoma cells $\text{Ins}(1,3,4,5)\text{P}_4$ induced Ca^{2+} release with an EC_{50} of $2 \mu\text{M}$ (Gawler *et al.*, 1990) and although the mechanism of $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Ca^{2+} -release is still a matter of controversy, Wilcox *et al* (1993), in studies using SH-SY5Y neuroblastoma cells have recently suggested that $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Ca^{2+} -mobilisation is mediated through the InsP_3 receptor.

Seewald *et al* (1990) found that 3-deoxy- $\text{Ins}(1,4,5)\text{P}_3$ was a full agonist in NIH 3T3 cells (no reported EC_{50}), and Kozikowski *et al* (1993*ab*), synthesized a series of deoxygenated analogues including 3-deoxy- $\text{Ins}(1,4,5)\text{P}_3$ which showed full agonism with an EC_{50} of $1.56 \pm 0.02 \mu\text{M}$ in SH-SY5Y neuroblastoma cells.

These results show that the 3-OH plays a relatively unimportant role in receptor binding and Ca^{2+} -mobilisation. However, the addition of a phosphate group at the 3-position [as in $\text{Ins}(1,3,4,5)\text{P}_4$] reduces the compounds ability to mobilise Ca^{2+} . Replacement with a phosphorothioate group, as in $\text{Ins}(3)\text{PS}(1,4,5)\text{P}_3$, further, but marginally reduces the ability for Ca^{2+} release.

4.1.4.4 Modification at the 4-position.

InsP_3 analogues modified exclusively at the 4-position were not available for this study. However, several analogues having multiple modifications have been modified at the 4-position, see later.

$\text{Ins}(1,3,5)\text{PS}_3$ does not possess a 4-phosphate moiety, but possesses 1-, 3- and 5-phosphorothioate groups. This analogue has no Ca^{2+} -mobilising activity, in permeabilised platelets, even at $10\mu\text{M}$, having an EC_{50} of $\geq 100\mu\text{M}$ at 20°C . Although this is a phosphorothioate analogue, the lack of activity indicates to the possible importance of this 4-phosphate for activity. Polokoff *et al* (1988) found that the parent compound, $\text{Ins}(1,3,5)\text{P}_3$ had low potency with an EC_{50} of $630\mu\text{M}$ in smooth muscle cells. It may be that phosphorothioate substitution of $\text{Ins}(1,3,5)\text{P}_3$, as in $\text{Ins}(1,3,5)\text{PS}_3$, will further reduce $\text{Ins}(1,3,5)\text{P}_3$ potency. However, it is known that the vicinal 4,5-bisphosphate or a pseudo D-4,5-bisphosphate is necessary for activity in other cell systems and the lack of activity of this compound is most likely due to this.

4.1.4.5 Modification at the 5-position.

InsP_3 analogues selectively modified at the 5-position were not available for this study, however, several analogues having multiple modifications have been modified at the 5-position, see later.

The natural product of dephosphorylation of InsP_3 by the 5-phosphatase enzyme, $\text{Ins}(1,4)\text{P}_2$, has been shown to be substantially weaker than InsP_3 in mobilising Ca^{2+} . This would suggest that it plays a minor role in Ca^{2+} -mobilisation *in vivo* (Potter, 1990*bc*)

D/L-*myo*-inositol 1,4-bisphosphate-5-phosphorothioate [$\text{Ins}(1,4)\text{P}_2(5)\text{PS}$] (Potter, 1990*b*; Safrany *et al.*, 1991*a*) was found to be a full agonist at the InsP_3 receptor and a potent mobiliser of Ca^{2+} from permeabilised SH-SY5Y neuroblastoma cells, being only 7 fold less potent than InsP_3 at mobilising Ca^{2+} , where the EC_{50} of InsP_3 is $0.11\mu\text{M}$ and for $\text{Ins}(1,4)\text{P}_2(5)\text{PS}$ is $0.8\mu\text{M}$.

4.1.4.6 Modification at the 6-position.

The role of the 6-OH, adjacent to the crucial vicinal 4,5-bisphosphate, was probed using 6-deoxy- $\text{Ins}(1,4,5)\text{P}_3$ and 6-deoxy- $\text{Ins}(1,4,5)\text{PS}_3$, where the 6-OH has been deleted in the first case and in addition, the phosphates replaced by phosphorothioates in the second case. Both of these analogues were weak as Ca^{2+} mobilizers, showing substantial loss in activity, 6-deoxy- $\text{Ins}(1,4,5)\text{P}_3$ releasing a maximum of 40% ^{45}Ca , with an EC_{50} of $\geq 30\mu\text{M}$ at 20°C , being at least 30-fold less potent than InsP_3 . Any activity expressed by 6-deoxy- $\text{Ins}(1,4,5)\text{P}_3$ is lost upon phosphorothioate replacement, for 6-deoxy- $\text{Ins}(1,4,5)\text{PS}_3$ releases maximally 8% Ca^{2+} at $100\mu\text{M}$.

6-deoxy- $\text{Ins}(1,4,5)\text{P}_3$ was found to be a full agonist in permeabilised SH-SY5Y neuroblastoma cells (Safrany *et al.*, 1991*b*; Potter and Nahorski, 1992; Potter, 1993) some 70-fold less potent (EC_{50} $6.4\mu\text{M}$) than InsP_3 with an EC_{50} of $6.4\mu\text{M}$ (InsP_3 EC_{50} $0.1\mu\text{M}$). However, in this study using permeabilised platelets 6-deoxy- $\text{Ins}(1,4,5)\text{P}_3$ was found to be a weak mobiliser of Ca^{2+} with an EC_{50} of

$\geq 30\mu\text{M}$. In contrast to our data, 6-deoxy-Ins(1,4,5)PS₃ was shown to be a partial agonist in SH-SY5Y cells (Safrany *et al.*, 1993), mobilising $\approx 50\%$ of the Ca²⁺ pool mobilised by InsP₃.

Thus, modification at the 6-position appears to be important in Ca²⁺-release, leading to substantial loss of activity.

4.1.4.7 Multiple modifications.

Ins(4)CO(3,5)P₂ is modified at the 1-, 3- and 4-position. Ins(4)CO(3,5)P₂ does not possess a 1-phosphate group of InsP₃, the 3-hydroxyl is occupied by a phosphate and its 4,5-positions are occupied by a methylene carboxylate and a phosphate group respectively. This compound shows no ability to mobilise Ca²⁺ in rabbit platelets. This is possibly due to the absence of the 1-phosphate. In addition, it does not antagonise InsP₃-induced Ca²⁺ release.

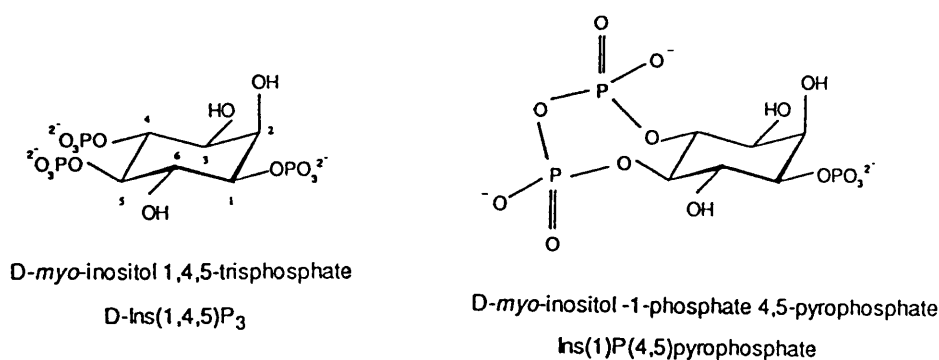


Figure 64. The structures of InsP₃ and Ins(1)P(4,5)pyrophosphate

Ins(1)P(4,5)pyrophosphate, in figure 64, is modified selectively at the vicinal 4,5-phosphates which are replaced by a pyrophosphate moiety. This analogue does not release Ca²⁺, even at high micromolar concentrations. In addition, it does not inhibit InsP₃-induced Ca²⁺-release, which may suggest that it does not

interact or bind to the InsP_3 receptor. The 4,5-pyrophosphate group is a rigid structure with a similar size to the two phosphate groups it has replaced.

However, the inability of this analogue to activate the receptor may be attributed to the rigid structure of the former, where the pyrophosphate group is held tightly in its position and is unable to rotate or move to achieve a desirable conformation for binding to the InsP_3 receptor. It was thought that InsP_3 may adopt a conformation, when bound to its receptor or in solution, where the vicinal 4,5-bisphosphate system chelates a metal ion. $\text{Ins}(1)\text{P}(4,5)\text{pyrophosphate}$ was synthesized to mimic this conformation in order to validate this idea. However, because this compound is inactive, it provides tentative evidence for a different conformation of InsP_3 when bound to the receptor. This is likely to be a conformation where the 4 and 5 phosphates are diametrically opposed as a result of ionic repulsion.

Inositol-1-phosphate, which does not possess the vicinal 4,5-phosphates, is unable to mobilise Ca^{2+} in permeabilised platelets, or to interfere with InsP_3 -induced Ca^{2+} -mobilisation. This lack of activity can be attributed to the absence of the 4,5-phosphates.

Thus, our experimental data with inositol phosphate analogues in which the vicinal 4,5-bisphosphate are absent, or modified, support previous findings with other cell systems. Similar attributes have been assigned to the 4,5-bisphosphate and the critical importance of the vicinal groups (Gawler *et al.*, 1991) and the enhancing effects of the 1-phosphate group of InsP_3 in receptor binding are now well established and appreciated (Potter, 1991; Nahorski and Potter, 1992; Potter, 1993).

The synthetic precursor of 6-deoxy-Ins(1,4,5)P₃, 6-deoxy-2,3-cyclohex-Ins(1,4,5)P₃ has a bulky cyclohexylidene group on the 2- and 3-OH groups and shows no activity in terms of Ca²⁺ release. The phosphorothioate derivative, 6-deoxy-2,3-cyclohex-Ins(1,4,5)PS₃, also has no activity. These synthetic precursors show complete loss of activity, in comparison to their parent compounds, 6-deoxy-Ins(1,4,5)P₃ and 6-deoxy-Ins(1,4,5)PS₃. This is possibly due to steric hindrance caused by the bulky cyclohexylidene group which may lead to ring distortion that may disrupt the correct conformation necessary for receptor binding. This is surprising, since modifications at the 2- and 3-positions do not effect potency and that these positions do not play a significant role in Ca²⁺-mobilisation. Alternatively, and most likely, the size of the 2,3-cyclohexylidene group may just be too large to be tolerated by the receptor.

Ins(1,3,4,6)P₄ was originally identified as a natural metabolite of InsP₃ produced from the actions of a 6-kinase on Ins(1,3,4)P₃ (Shears *et al.*, 1987; Stephens *et al.*, 1990). In platelets, Ins(1,3,4,6)P₄ was found to be a full agonist, and to mobilise Ca²⁺ with an EC₅₀ of $9.67 \pm 0.5 \mu\text{M}$, this is some 8-fold less potent than InsP₃. On this basis it seems unlikely that it will have any significant role in cellular signalling and Ca²⁺ release. However, this polyphosphate was shown to be a partial agonist of high efficacy in SH-SY5Y neuroblastoma cells, mobilising 80- 90% of the Ca²⁺ pool mobilised by InsP₃, see Safrany *et al* (1993). The phosphorothioate analogue, Ins(1,3,4,6)PS₄, was also investigated in rabbit platelets, and was found to be a weak full agonist, mobilising Ca²⁺ with an EC₅₀ of $\geq 30 \mu\text{M}$. This is consistent with our previous findings that substitution of a phosphate for a phosphorothioate reduces the compounds activity for Ca²⁺-release.

When examining the chemical structure of Ins(1,3,4,6)P₄, it is not immediately apparent why it should be active at all. Ins(1,3,4,6)P₄ does not nominally

possess the essential vicinal 4,5-bisphosphate structure, nor does it possess a free 6-OH, which in this case is phosphorylated.

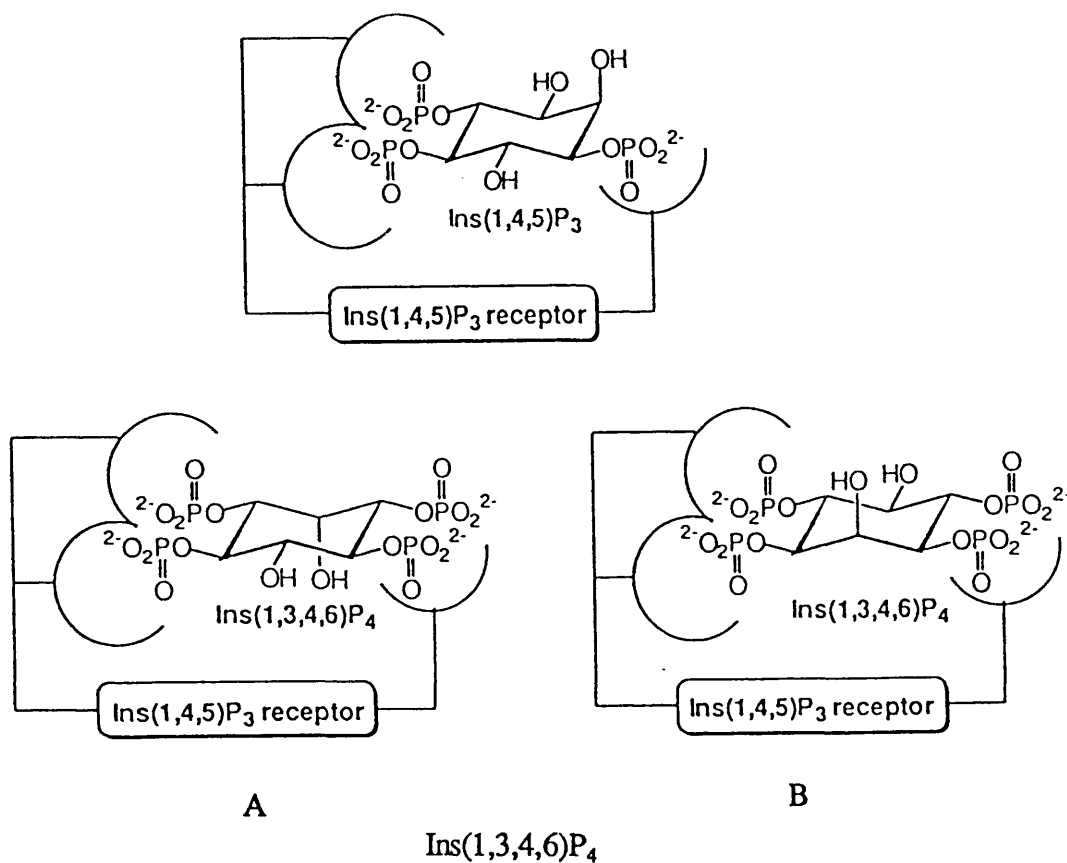


Figure 65. Alternative binding conformations for Ins(1,3,4,6)P₄

However, it is possible to envisage two potential binding conformations for Ins(1,3,4,6)P₄ at the InsP₃ receptor, A and B in figure 65. Both conformations mimic a number of important recognition features, namely, the 4,5-bisphosphate, the 1-phosphate group and the 6-hydroxyl group.

In conformation A, the 1- and 6-phosphates of Ins(1,3,4,6)P₄ resemble the vicinal 4,5-bisphosphate of InsP₃, and the 4-phosphate is in the position of the 1-phosphate of InsP₃. In conformation B, again, the 6- and 1-phosphates of Ins(1,3,4,6)P₄ resemble the vicinal 4,5-bisphosphate of InsP₃. However, in this

case, it is the 3-phosphate that is in the position of the 1-phosphate of InsP₃, see figure 65.

Both conformations contain an extra phosphate at the 2-position of InsP₃ is apparent from figure 65. However, modifications at the 2-position do not have a significant effect on Ca²⁺-mobilisation, as discussed earlier and we know that *scyllo*-Ins(1,2,4,5)P₃ is highly potent. In addition, the conformation of the 3-hydroxyl (in the first binding conformation A) and the conformation of the 6-hydroxyl (in the second binding conformation B) are altered, both being axial, rather than equatorial as in the case of InsP₃. Thus, this compound can mimic the essential features necessary for InsP₃ receptor binding, namely the D-1,4,5-phosphates and the 6-OH.

On the basis that Ins(1,3,4,6)P₄ was found to be a partial agonist in neuroblastoma cells, with high intrinsic activity, it was thought that deletion of either of the 3- or 4-phosphates of Ins(1,3,4,6)P₄, and the further substitution of the phosphates with phosphorothioates may give rise to a partial agonist with lower intrinsic activity (see section on partial agonists).

Deletion of the 3-phosphate gives rise to D-Ins(1,4,6)P₃ and deletion of the 4-phosphate gives rise to L-Ins(1,3,4)P₃. According to this rationale, two such analogues (as their racemic mixtures) were synthesised, along with their phosphorothioate analogues (Mills et al., 1993).

D/L-Ins(1,3,4)P₃ was a full agonist with an EC₅₀ of $\geq 30\mu\text{M}$ at 4°C. This is in agreement with Irvine *et al.*, (1986) who report an EC₅₀ of 9 μM for the D-isomer, but in marked contrast to studies by Strupish *et al.*, (1988), where D-Ins(1,3,4)P₃ is found to be virtually inactive in Swiss 3T3 cells.

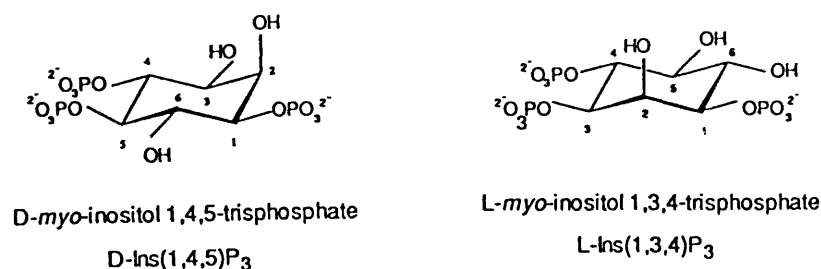


Figure 66. The structures of InsP₃ and L-Ins(1,3,4)P₃

InsP₃ L-Ins(1,3,4)P₃ comparison of -OH

2 axial	4 equatorial	Different
3 equatorial	5 equatorial	Same
6 equatorial	2 axial	Different

Table 6. Comparison of the position and orientation of the hydroxyls of InsP₃ and L-Ins(1,3,4)P₃

Polokoff *et al* (1990) also found D/L-Ins(1,3,4)P₃ to be some 400-fold less potent than InsP₃ (DiPersio *et al.*, 1988; Gawler *et al.*, 1990). Hirata *et al* (1993) have recently evaluated the Ca²⁺-release ability of the resolved enantiomers of Ins(1,3,4)P₃ and Ins(1,4,6)P₃. They conclude that the activities exhibited by these two compounds are due to L-Ins(1,3,4)P₃ and D-Ins(1,4,6)P₃ respectively. Furthermore, upon examination of the structure of L-Ins(1,3,4)P₃, it is possible to see a way of arranging the phosphates in a pseudo D-1,4,5-orientation as explained previously. The same is not evident for the D-enantiomer. Thus, it seems most likely that the activity exhibited by D/L-

Ins(1,3,4)P₃ in our cell system is due to the L-enantiomer, see figure 66. Table 6 shows the orientation of the ring hydroxyls in comparison to InsP₃.

We have shown the racemic mixture of Ins(1,3,4)P₃ to be a weak agonist. However, it is possible that upon phosphorothioate substitution, partial agonist activity may become apparent, see Ins(1,4,6)P₃ and Ins(1,4,6)PS₃.

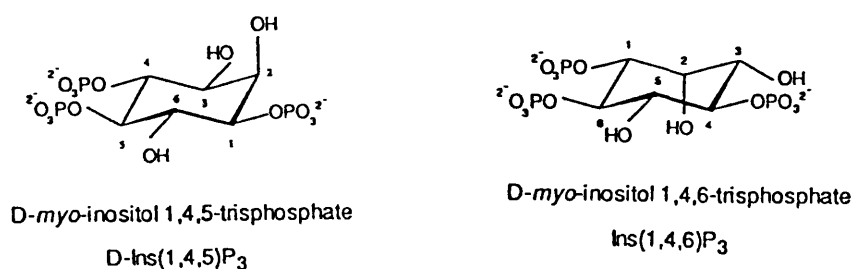


Figure 67. The structures of InsP₃ and D-Ins(1,4,6)P₃

Irvine *et al* (1986) report an EC₅₀ of 9 μM for the D-isomer of Ins(1,3,4)P₃. This is not in agreement with other reports on the activity of this analogue, discussed above, along with our data. It is possible, because the source of this analogue was not synthetic, that D-Ins(1,3,4)P₃ was contaminated with InsP₃, Ins(1,3,4,5)P₄ and/or L-Ins(1,3,4)P₃. Under these circumstances, the activity observed by this analogue could be due to any of these contaminating compounds.

Ins(1,4,6)P₃ (Mills *et al.*, 1993), see figure 67, was found to be a full agonist with an EC₅₀ of 2.07 ± 0.08 μM at 4°C, and is 11-fold weaker than InsP₃ (InsP₃ EC₅₀ of 0.18 μM at 4°C). Therefore, despite the differences in the orientation of the pseudo 2- and 3-OH of Ins(1,4,6)P₃, see table 7, the compound is still able to mobilise Ca²⁺ indicating that these two hydroxyls play a minor role in InsP₃

binding and activation. It was also found that substitution of the phosphates with phosphorothioates rendered this analogue a partial agonist, see section on partial agonists.

<u>InsP₃</u>	<u>Ins(1,4,6)P₃</u>	<u>comparison of -OH</u>
2 axial	3 equatorial	Different
3 equatorial	2 axial	Different
6 equatorial	5 equatorial	Same

Table 7. Comparison of the position and orientation of the hydroxyls of InsP₃ and Ins(1,4,6)P₃

Both L-Ins(1,3,4)P₃ and D-Ins(1,4,6)P₃ possess a pseudo D-1,4,5-orientation. But, are modified at the pseudo 2- and 6-OH for L-Ins(1,3,4)P₃ (table 6), and pseudo 2- and 3-OH for Ins(1,4,6)P₃ (table 7). Both these compounds were full agonists. This suggests that the modifications at the 2-, 3- and 6-hydroxyls do not significantly effect Ca²⁺-mobilisation. Indeed, this is in agreement with the data of Wilcox *et al* (1992) and Hirata, *et al* (1933) where it was found that both 3- and 6-OH are important in receptor binding, the 6-OH appears to be more important in Ca²⁺-mobilisation. Kozikowski *et al* (1993ab) also provide evidence for the crucial role for 6-OH.

In conclusion, then, we can see that in our system both L-Ins(1,3,4)P₃ and Ins(1,4,6)P₃ were found to be full agonists. However, phosphorothioate substitution of Ins(1,4,6)P₃ has provided the best example of a partial agonist with low intrinsic activity, see section on partial agonists. This is in accordance with the original rationale involved in the synthesis of these analogues.

The synthetic precursor of $\text{Ins}(1,2,4,5)\text{P}_4$, $\text{Ins}(3,6)\text{Bz}_2(1,2,4,5)\text{P}_4$ does not mobilise Ca^{2+} . This may be due to the presence of two bulky benzoyl protecting groups on the 3- and 6-positions. It is possible that these protecting groups are too large for the receptor to tolerate them. Alternatively, these groups may produce ring distortion, preventing receptor binding.

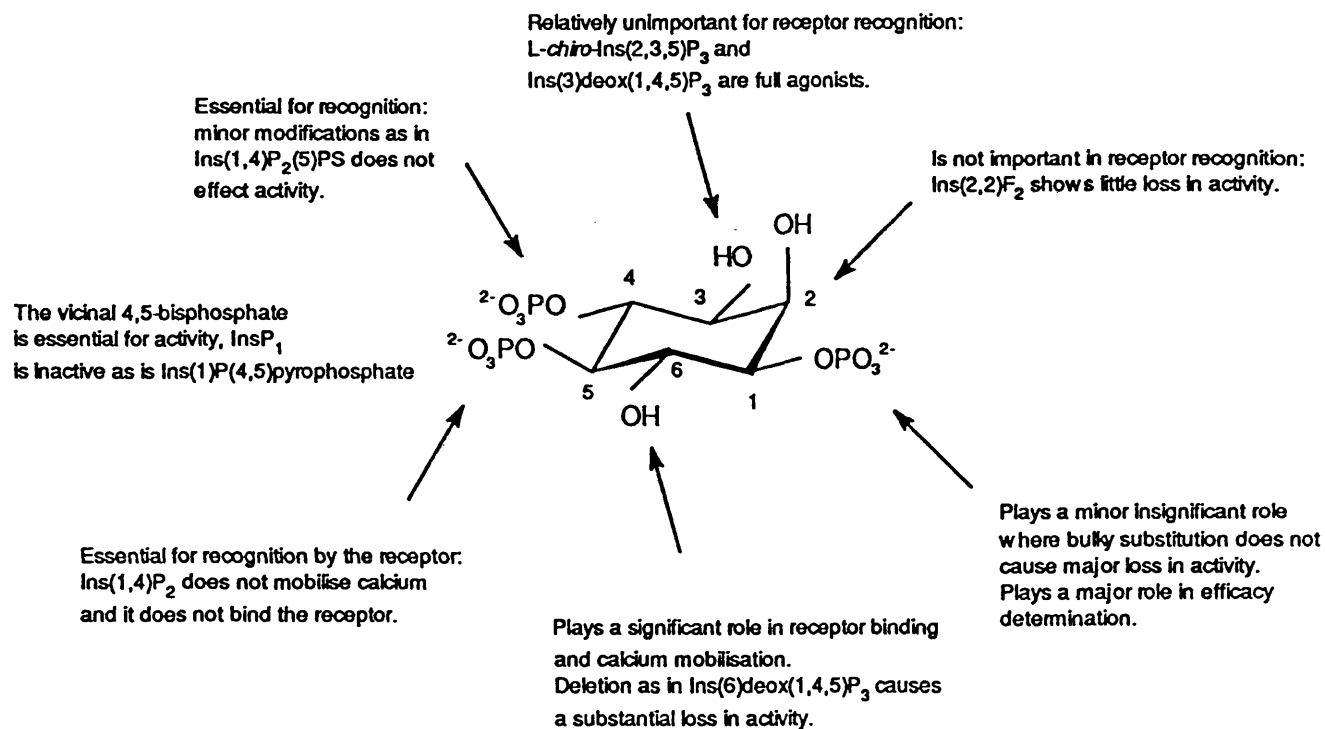


Figure 68. Summary of the structural requirements for activity at the platelet InsP_3 receptor

Analogue	Platelets	Cell system
D-InsP ₃	0.18 ± 0.1 μM	0.12 μM in SH-SY5Y (Safrany <i>et al.</i> , 1992; Wilcox <i>et al.</i> , 1993)
	0.4 μM in membranes (Adunyah and Dean, 1985)	0.3 μM Swiss 3T3 cells (Irvine <i>et al.</i> , 1986)
L-InsP ₃	inactive	inactive in SH-SY5Y (Strupish <i>et al.</i> , 1988); inactive in swiss 3T3 cells (Giese and Kirchner, 1988)
Ins(1,4,5)PS ₃	0.8 ± 0.1 μM	2.5 μM in SH-SY5Y (Safrany <i>et al.</i> , 1991)
Ins(2,2)F ₂ (1,4,5)P ₃	0.6 ± 0.09 μM	0.41 μM in SH-SY5Y (Safrany <i>et al.</i> , 1990; Safrany <i>et al.</i> , 1992)
L-chiro-Ins(2,3,5)P ₃	1.9 ± 0.1 μM	1.4 ± 0.3 μM in SH-SY5Y (Safrany <i>et al.</i> , 1992)
Ins(1,3,4,5) ₄	≥ 10 μM	2 μM (Gawler <i>et al.</i> , 1990; Ghiggeri <i>et al.</i> , 1984; Kaiser <i>et al.</i> , 1981) in SH-SY5Y; ≥ 30 μM in swiss 3T3 cells (Irvine <i>et al.</i> , 1986)
6-deoxy-Ins(1,4,5)P ₃	≥ 30 μM	6.4 μM in SH-SY5Y (Safrany <i>et al.</i> , 1991)
Ins(1)P(4,5)pyroP	inactive	inactive in SH-SY5Y (Noble <i>et al.</i> , 1992)
D/L-Ins(1,3,4)P ₃	≥ 30 μM	120 μM in smooth muscle cells (Polokoff <i>et al.</i> , 1988) ≥ 30 μM in RBL cells (Hirata <i>et al.</i> , 1993)
D/L-Ins(1,4,6)P ₃	≥ 30 μM	≥ 30 μM in RBL cells (Hirata <i>et al.</i> , 1993)

Table 8. Comparison of analogues studied in permeabilised platelets with other cells.

Analogue	Platelets	Cell system
Ins(1,3,4,6)P ₄	9.67 ± 0.5 μM	Partial agonist in SH-SY5Y (Gawler <i>et al.</i> , 1991)
6-deoxy-Ins(1,4,5)PS ₃	≥ 30 μM	partial agonist in SH-SY5Y (Safrany <i>et al.</i> , 1993)
L-chiro-Ins(2,3,5)PS ₃	partial agonist	partial agonist in SH-SY5Y (Safrany <i>et al.</i> , 1993)
Scyllo-Ins(1,2,4,5)PS ₄	partial agonist	not known
Ins(1,4,6)PS ₃	partial agonist	not known

Table 8. Comparison of the activities of the partial agonists found in platelets with different cell systems.

4.1.5 Partial agonists.

Partial agonism was displayed by several of the compounds studied, these being *scyllo*-Ins(1,2,4,5)PS₄, *L-chiro*-Ins(2,3,5)PS₃ and D/L-Ins(1,4,6)PS₃. Interestingly, *scyllo*-Ins(1,2,4,5)P₄, *L-chiro*-Ins(2,3,5)P₃ and Ins(1,4,6)P₃, the parent polyphosphates of each phosphorothioate analogue were all full agonists. Of the three phosphorothioates, only Ins(1,4,6)PS₃ caused a dose dependent inhibition of InsP₃-induced Ca²⁺ release with 100 μM Ins(1,4,6)PS₃ producing 50% inhibition.

In SH-SY5Y cells, receptor binding has been characterised, and it appears that the introduction of phosphorothioate groups causes a substantial reduction in agonist efficacy with only a small reduction in the receptor affinity, and because [³⁵S]Ins(1,4,5)PS₃ labels two populations of sites with similar affinity one of which is the InsP₃ receptor and the second is a site that displays low affinity for InsP₃ (Safrany *et al.*, 1993). It is conceivable that InsP₃ receptors in platelets also possess these two affinity sites. As a result, it is tentatively suggested that the two partial agonists that show no antagonistic activities i.e. *scyllo*-Ins(1,2,4,5)PS₄ and *L-chiro*-Ins(2,3,5)PS₃, are acting through this second low affinity site mobilising Ca²⁺ and having no effect on the high affinity InsP₃ receptor. Thus, upon investigation of the effect of *scyllo*-Ins(1,2,4,5)PS₄ and *L-chiro*-Ins(2,3,5)PS₃ on InsP₃-induced Ca²⁺-release, inhibition is not observed.

It is known that phosphorothioate substitution leads to changes in molecular size, hydrophobicity and charge distribution, any or all of which may contribute to producing a less than maximal response. Thus, we suggest that phosphorothioate substitution coupled with another perturbation are required for a compound to exhibit partial agonism. Partial agonist activity may be generated

by a suboptimal binding interaction produced by a D-1,4,5-trisphosphorothioate motif, in combination with another structural perturbation, such the presence of an additional equatorial 2-phosphorothioate group in *scyllo*-Ins(1,2,4,5)PS₄, or the presence of an 3-axial hydroxyl of *L-chiro*-Ins(2,3,5)PS₃, or the equatorial 2-OH and axial 3-OH of Ins(1,4,6)PS₃, see figure 68.

It should be noted that for the second messenger cAMP phosphorothioate substitution has generated competitive cAMP antagonists, see Safrany *et al* (1993). These antagonists are Rp-cAMPS and cAMPS₂ (Parker Bothelo *et al.*, 1988,*ab*) reviewed in Potter (1992).

Upon examining the structures of the three inositol phosphate partial agonists, it appears that they share a high degree of similarity between each other and with InsP₃, see figure 69. *Scyllo*-Ins(1,2,4,5)PS₄ does have 1-, 4-, and 5-phosphorothioates. However, *L-chiro*-Ins(2,3,5)PS₃ and Ins(1,4,6)PS₃ do not nominally possess phosphates at the 1-, 4- and 5- positions, analogues to InsP₃, it is nevertheless possible to envisage binding conformations of these two compounds in which do they have phosphorothioates at these positions, see figure 69. Of the three partial agonists, *scyllo*-Ins(1,2,4,5)PS₄ possessed the highest intrinsic activity, releasing $61.7 \pm 11\%$ ⁴⁵Ca. This was followed by *L-chiro*-Ins(2,3,5)PS₃, which possessed a lower intrinsic activity, releasing $42.3 \pm 6\%$ ⁴⁵Ca, and D/L-Ins(1,4,6)PS₃ being the partial agonist with the lowest intrinsic activity, releasing $21.1 \pm 4\%$ ⁴⁵Ca.

Ins(1,4,6)PS₃ (3- 100μM) produces 50% inhibition at 100μM, and it appears that inhibition of the InsP₃ response is only occurring at concentrations of 10μM and above. This is consistent with the dose response curve of Ins(1,4,6)PS₃

where the curve appears to be in two phases. A low level of release is observed at concentrations up to $10\mu\text{M}$. At concentrations above $10\mu\text{M}$,

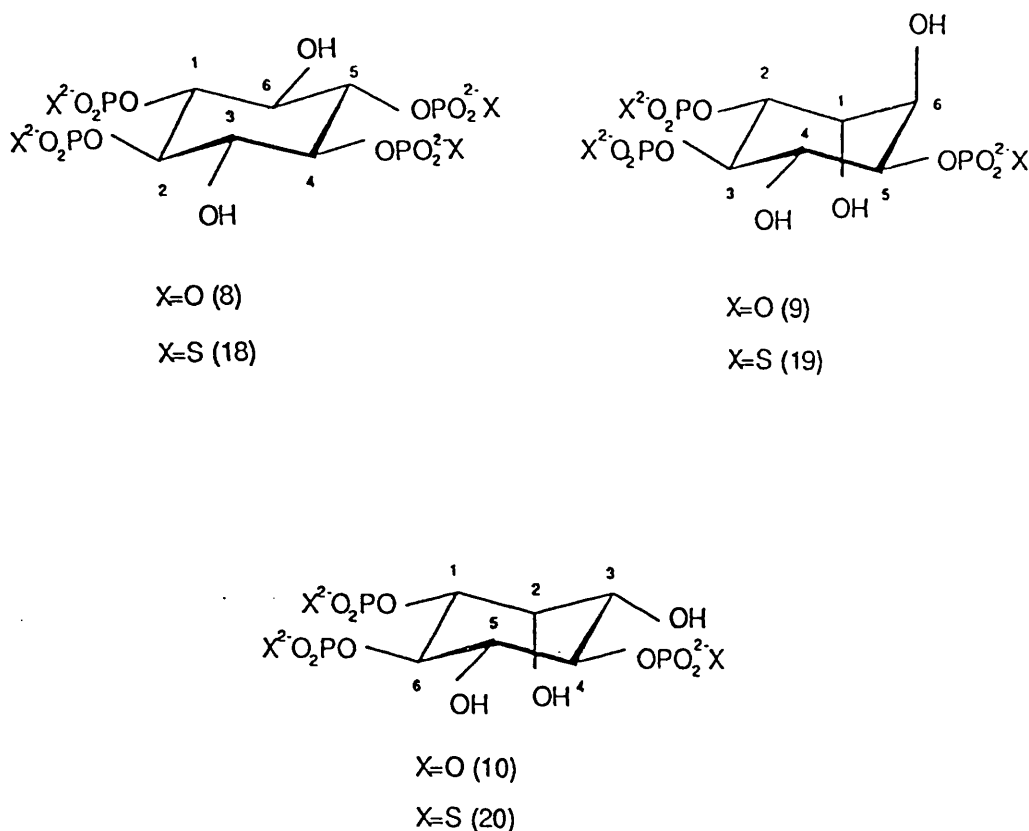


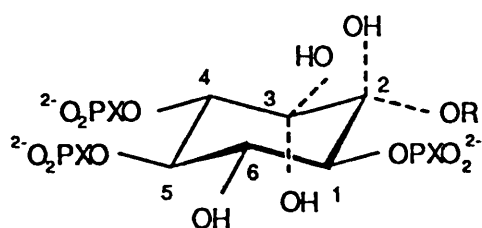
Figure 69. The chemical structures and numbering of *scyllo*-Ins(1,2,4,5) P_4 (8); *scyllo*-Ins(1,2,4,5) PS_4 (18); *L-chiro*-Ins(2,3,5) P_3 (9); *L-chiro*-Ins(2,3,5) PS_3 (19); Ins(1,4,6) P_3 (10) and Ins(1,4,6) PS_3 (20).

a second phase of higher release is observed plateauing out at $100\mu\text{M}$ and it is at these concentrations that an inhibitory effect is observed. Alternatively, an inhibitory response may be occurring at the lower concentrations of $0.03\text{--}3\mu\text{M}$, but is at such a weak level, that its effects are within the limits of error.

Thus, in order to synthesize further analogues that are partial agonists with even lower intrinsic activity, or even antagonists, it appears that all D-1,4-5-positions of InsP₃ need to be occupied by phosphorothioates, see figure 70. In addition, as mentioned above, it is possible the further modifications at the pseudo 2- and 3- positions of InsP₃, as seen in all three of the partial agonists, confer low intrinsic activity.

In this study 6-deoxy-Ins(1,4,5)PS₃ did not show partial agonism or affect InsP₃-induced ⁴⁵Ca release. However, the EC₅₀ for this analogue was too high and this may mask any partial agonist activity present. This is in contrast with reports using SH-SY5Y neuroblastoma cells (Safrany *et al.*, 1993).

Further, Ins(1,3,4,6)P₄ which was also found to be a partial agonist in SH-SY5Y neuroblastoma cells (Potter and Nahorski, 1993; Gawler *et al.*, 1991) was found to be a full agonist in permeabilised platelets (this thesis). An interpretation of the differences between the activities observed in platelets and neuroblastoma cells is the existence of tissue specific receptor populations (see summary of discussion).



R= H or PXO_2^{2-}

X= S or any other group at the 1-, 4- or 5- positions

----- hydroxyls at these positions can have either orientation, axial or equatorial

Figure 70. The proposed structural requirements for partial agonist activity.

4.1.6 InsP₃-receptor antagonists and Inhibitors of InsP₃-induced Ca²⁺-release.

4.1.6.1 Heparin.

Heparin is a polysulphated polysaccharide with a molecular weight between 6000 and 20,000, depending on its origin and preparation. It is a competitive reversible antagonist of InsP₃ binding at the InsP₃-receptor (Ghosh *et al.*, 1986). In saponin permeabilised platelets, heparin up to 100µg/ml had no effect on InsP₃-induced Ca²⁺-release. In this study two heparins, one with high molecular weight and the second with 3000 molecular weight were used and both were ineffective in inhibiting InsP₃-induced Ca²⁺ release in permeabilised platelets.

This can be explained by the fact that stimulated platelets secrete antiheparin proteins. As early as 1955 Deutsch introduced the term platelet factor 4 (PF4) to describe a platelet protein with antiheparin activity (Rucinski *et al.*, 1979). In addition, two further proteins secreted by platelets were found to possess weak antiheparin activity, these being β-thromboglobulin (βTG) and low-affinity platelet factor 4 (LA-PF4) (Rucinski *et al.*, 1979). These antiheparin proteins act to inactivate heparin activity, and it can be envisaged that upon heparin incubations, these proteins will act to neutralise heparin and upon subsequent InsP₃-stimulation Ca²⁺ release will occur as usual.

The only study on the effect of heparin on InsP₃-induced calcium mobilisation in permeabilised platelets was that of Rao and colleagues (Rao *et al.*, 1991), and they found that high molecular weight (2- 15 kDa) heparins have no inhibitory effects and only one of six low molecular weight heparins (4- 6 kDa, sigma H5640) caused a significant inhibition of InsP₃-induced Ca²⁺-mobilisation. They attribute this lack of inhibition to the way permeabilisation was achieved, using

low saponin concentration. However, other studies have used saponin to permeabilise cells (Ghosh *et al.*, 1986) and this appears not to interfere with the inhibitory effects of heparin reported as having an IC_{50} of $5\mu\text{g/ml}$. However, they do report that sensitivity to heparin increases with decreasing the molecular size.

In addition to inhibiting InsP_3 binding, heparin also inhibits InsP_3 3-kinase activity, but it has no effect on InsP_3 5-phosphatase activity or $\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase activity. In addition, it inhibits $\text{Ins}(1,3,4,5)\text{P}_4$ binding and its ability to mobilise Ca^{2+} (Taylor and Richardson, 1991) and these effects severely limit the use of heparin as an InsP_3 receptor antagonist.

4.1.6.2 Decavanadate.

Decavanadate, a decameric vanadate cluster, inhibits InsP_3 -induced Ca^{2+} -mobilisation (Föhr *et al.*, 1989). In permeabilised rabbit platelets, decavanadate caused a dose dependent inhibition of InsP_3 -induced ^{45}Ca -release with an IC_{50} of $7.82 \pm 0.4\mu\text{M}$.

It has been found to inhibit competitively InsP_3 -binding and to inhibit InsP_3 -induced Ca^{2+} -release (IC_{50} of $5\mu\text{M}$) (Strupish *et al.*, 1991*ab*; Föhr *et al.*, 1991). However, decavanadate was found to be non-specific in its inhibitory activity of InsP_3 -binding (Strupish *et al.*, 1991*ab*). Decavanadate was also found to inhibit InsP_3 3-kinase and $\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase (Strupish *et al.*, 1991) in permeabilised SH-SY5Y neuroblastoma cells as well as inhibiting the specific binding of $\text{Ins}(1,3,4,5)\text{P}_4$ to cerebellar membranes and its ability to mobilise Ca^{2+} from cerebellar microsomes, (Challis *et al.*, 1991; Strupish *et al.*, 1991*ab*).

Although decavanadate and heparin are potent and competitive antagonists at the InsP_3 receptor, they possess an array of other actions which limits their use as tools in investigating the InsP_3 signalling pathway and clearly small-molecule antagonists are required.

4.1.6.3 Benzene 1,2,4-trisphosphate.

Recently, a very loose structural analogue of InsP_3 , benzene 1,2,4-trisphosphate (BzP_3) was shown to interact with the InsP_3 receptor where it inhibited InsP_3 -binding to adrenal cortex microsomes with an IC_{50} of $428\mu\text{M}$ and an 80% inhibition at $500\mu\text{M}$ (Poitras *et al.*, 1993). In permeabilised rabbit platelets, we found that BzP_3 had no inhibitory effect on InsP_3 -induced Ca^{2+} -mobilisation at concentrations up to $500\mu\text{M}$. This lack of activity, on the InsP_3 response, could be due to our different source of this compound, or that tissue specific receptors are present.

4.1.7 The InsP_3 receptor.

Limited information is available at present concerning the conformation(s) adopted by InsP_3 in solution, and there is no information relating to the conformation of this molecule in the bound state. An early study on pH effects on the conformation of InsP_6 in solution demonstrated that at neutral and alkaline pH, InsP_6 adopts the expected conformation of five equatorial and one axial phosphate groups, whereas at acidic pH the cyclohexane ring "flips" to give reverse conformation with one equatorial and five axial phosphate substituents stabilised by intramolecular hydrogen-bonding interactions (Challiss *et al.*, 1991). This unusual conformation may also be adopted at acidic pH by other highly charged phosphorylated inositol derivatives, and the likelihood of

this occurrence will presumably increase with increasing the degree of phosphorylation. However, it is unlikely that this will be the case with InsP_3 , due to the small number of phosphate groups in comparison to InsP_6 . A more recent study of pH effects on the solution conformation of InsP_3 did not support this idea (Lindon *et al.*, 1987). Thus, the distinct pH-dependence of InsP_3 -receptor binding can not be explained by the predicted physical properties of the ligands. Furthermore, the difference may reside in the protein conformation and ionisation of titratable groups within the ligand-binding site of the receptor (Challiss *et al.*, 1991).

The initial cloning of the cDNA for the InsP_3 receptor from mouse cerebellum has provided a substantial image of the receptor structure (Furuichi *et al.*, 1989), and subsequent studies from other tissues has shown that the sequence of the receptor is highly conserved (Mikoshiba, 1993). Subsequent transfection of cDNA (Furuichi *et al.*, 1989) and reconstitution (Ferris *et al.*, 1989; Maeda *et al.*, 1991; Kamata *et al.*, 1992) experiments suggested that the cDNA actually encodes for a protein containing both the ligand binding site and Ca^{2+} -channel activity. From these data it was concluded that the receptor is a Ca^{2+} channel (Nakade *et al.*, 1991). The primary sequence of the receptor shares no homology with the voltage-operated Ca^{2+} channels on the plasma membrane (Furuichi *et al.*, 1989), but shares a significant homology with the ryanodine receptor, for review see (Mikoshiba *et al.*, 1993; Mikoshiba, 1993).

Patterns of Ca^{2+} release by InsP_3 suggested an intracellular localisation of the InsP_3 receptor in non-mitochondrial stores associated with microsomal fractions. However, there has been considerable debate over the intracellular localisation of this receptor (Mikoshiba, 1993; Mikoshiba *et al.*, 1993). The InsP_3 receptor has been found to be present in multiple subcellular areas (Sharp *et al.*, 1992)

and one study suggests that the receptor associates with the plasma membrane, suggesting the presence of receptor subtypes (Sharp *et al.*, 1992). In addition, neuronal and non-neuronal receptors have been shown to exist (Danoff *et al.*, 1991). Further, biochemical data demonstrated that InsP₃ binding has different characteristics in different tissues (Guillemette *et al.*, 1988; Rossier and Putney, 1991; Südhof *et al.*, 1991), and that different receptors may be expressed in different subcompartments (Südhof *et al.*, 1991).

Three subtypes of the InsP₃ receptor from different genes have been identified (Südhof *et al.*, 1991; Blondel *et al.*, 1993; Mikoshiba *et al.*, 1993; Mikoshiba, 1993) all having different affinities for InsP₃. Therefore it is possible that InsP₃-mediated Ca²⁺ release is directed by various forms of InsP₃ receptor (from the different genes) and these may possibly have different functional properties in Ca²⁺ release. There is then the possibility that intracellular Ca²⁺ signalling may be regulated in different ways dependent on different InsP₃ receptor transduction pathways, thus giving rise to multiple pathways for Ca²⁺-release.

4.1.7.1 Receptor regulation.

The InsP₃ receptor is regulated by pH, ATP, calcium and magnesium (Taylor and Richardson, 1991; Van Delden *et al.*, 1993), and phosphorylation by PKA (Hajnóczky *et al.*, 1993), PKC and Ca²⁺/calmodulin-dependent protein kinase II (Taylor and Richardson, 1991; Mikoshiba, 1993). Further, the membrane associated cytoskeletal protein, ankyrin, is also thought to play a role in receptor regulation where it was found to inhibit InsP₃ binding and InsP₃-mediated Ca²⁺ release (Bourguignon *et al.*, 1993). ATP enhances InsP₃-dependent Ca²⁺ release (Mikoshiba *et al.*, 1993), and ATP binding sites are located near the receptor phosphorylation sites (Ferris *et al.*, 1992; Mikoshiba, 1993). Ca²⁺ is known to

play a pivotal role in InsP₃ binding, and InsP₃-induced Ca²⁺ flux shows a bell shaped curve depending on the Ca²⁺ concentration (Danoff *et al.*, 1988; Varney *et al.*, 1990). A recent report suggest that receptor activation which causes elevations in [Ca²⁺]_i might result in the degradation of the N-terminal, InsP₃-binding part of the receptor mediated through an enzyme belonging to the calpain family (Magnusson *et al.*, 1993).

PKA, PKC and CaM Kinase II phosphorylate the receptor, the former being the most efficient (Ferris *et al.*, 1991). These enzymes phosphorylate three distinct serines (Ferris *et al.*, 1991). PKA phosphorylation is associated with an alteration in the potency of InsP₃ in releasing Ca²⁺ and may increase the sensitivity of the calcium stores to InsP₃ (Ferris *et al.*, 1992; Hajnoczky *et al.*, 1993). Furthermore, a recent report suggests that the purified and reconstituted receptor autophosphorylates on serine and displays protein kinase-like activity (Ferris *et al.*, 1992) and the physiological role for autophosphorylation is thought to be in modifying the response of the receptor to InsP₃, Ca²⁺ and ATP (Ferris *et al.*, 1992). The InsP₃ receptor is the first ion channel protein complex to display serine autophosphorylation activity and it may be the first receptor of any sort for which serine kinase activity on other substrates and on the receptor itself to be recognised. Although the InsP₃ receptor does not appear to have a full protein kinase domain (Ferris *et al.*, 1992), it does bind ATP with high affinity making it an ideal candidate for autophosphorylation.

4.1.8 Additional compounds investigated.

4.1.8.1 cyclic ADP-ribose (cADPR).

cADPR was first shown to mobilise Ca^{2+} in sea urchin eggs (Lee *et al.*, 1989). Subsequently, cADPR has been found to be the endogenous activator of the non-skeletal ryanodine receptor (Galione, 1992; Meszaros *et al.*, 1993) and in addition the ryanodine receptor has been cloned (Otsu *et al.*, 1990) and like the InsP_3 -receptor has been found to possess an intrinsic Ca^{2+} channel (Llach, 1985; Chen *et al.*, 1993).

In this study cADPR and its precursor ADPR were both inactive in releasing Ca^{2+} in permeabilised platelets. This could be due to the possibility that platelets do not possess ryanodine receptors, as to date no evidence is available that suggests the presence of ryanodine receptor in platelets. Alternatively, cADPR may be rapidly hydrolysed in platelets, or that the receptors are present only in low numbers. Indeed, cADPR is very rapidly hydrolysed in cells as might be expected of a molecule with such potent Ca^{2+} -mobilising activity (Galione, 1992). In addition, a cADPR-hydrolysis enzyme activity has been found in rabbit brain extract (Rusinko and Lee, 1989).

4.1.8.2 Phorbol esters (PMA) and diacylglycerol (DAG).

It has been reported that phorbol esters, which are direct activators of isozymes of PKC, except PKC zeta, attenuate InsP_3 -induced Ca^{2+} -release in electroporated rat pancreatic acinar cells (Arita *et al.*, 1992). King and Rittenhouse suggest this is due to the enhancement of InsP_3 hydrolysis in thrombin stimulated platelets (King and Rittenhouse, 1989). However, in human

and rabbit platelets, activation of PKC inhibits receptor induced InsP_3 formation and Ca^{2+} elevation. Furthermore, inhibitors of PKC markedly stimulate receptor induced formation of InsP_3 and prolong the elevation of cytosolic Ca^{2+} (Poll and Westwick 1986; Murphy *et al.*, 1991; Murphy *et al.*, 1992).

Yoshida and Nachmias (Suzuki *et al.*, 1990) report that pretreatment of platelets with PMA before saponin treatment increases ATP-dependent ^{45}Ca -uptake and that most of the sequestered ^{45}Ca is released by InsP_3 or ionomycin.

In permeabilised rabbit platelets pretreatment with PMA or DAG had no effect on InsP_3 -induced Ca^{2+} -mobilisation at concentrations up to $100\mu\text{M}$ and 100nM respectively, and they had no capacity to mobilise ^{45}Ca alone. Activation of endogenous PKC by DAG is thought to regulate InsP_3 levels (Lin *et al.*, 1990; Taylor, 1992; Thomas *et al.*, 1992; Cobbold *et al.*, 1993). Many authors have proposed that oscillations in InsP_3 levels are controlled at the level of the receptor/signal transduction apparatus through a cyclical activation of DAG-dependent PKC leading to feedback inhibition of InsP_3 formation by the inactivation of PLC which also results in lowering DAG levels. In addition, Brass and Laposata (Brass and Laposata, 1987) have found that OAG, a synthetic analogue of DAG, caused ^{45}Ca -release from the dense tubular system. However, they found that the phorbol esters TPA did not duplicate this effect and they conclude that it is unlikely that the effects they observed from OAG were mediated through PKC.

It appears from the conflicting data available that the role of DAG in InsP_3 -induced Ca^{2+} -release is still in contention, attenuating release in some cells whilst enhancing it in others. However, in our system, PMA and DAG did not mobilise Ca^{2+} or influence InsP_3 -induced Ca^{2+} -mobilisation.

4.2 Metabolism of [^3H]InsP₃ and the effect of some enzyme inhibitors.

[^3H]InsP₃ metabolism was examined at 4°C, 20°C and 37°C. Dramatically different metabolic profiles were observed at these three temperatures. At 4°C, [^3H]InsP₃ is conserved to such a great extent, that it is still present at 10 minutes. In addition, at 4°C, very little Ins(1,3,4,5)P₄ and Ins(1,3,4)P₃ are formed throughout the 10 minute period. In marked contrast, at 20°C, substantial amounts of Ins(1,3,4,5)P₄ are formed after 1 minute, and Ins(1,3,4)P₃ being formed after 3 minutes. However, at 37°C, [^3H]InsP₃ metabolism is completed by 3 minutes. Furthermore, at 37°C, Ins(1,3,4,5)P₄ is not detected at all, Ins(1,3,4)P₃ is formed, but only as a small proportion and by 10 minutes, only Ins(4)P₁ is left.

The temperature dependence of InsP₃ metabolism is a reflection of enzyme activity. At 4°C, enzyme activity is minimal thus maintaining higher levels of [^3H]InsP₃ over a longer time scale. Whereas, enzyme activity is greater at 37°C, thus reducing [^3H]InsP₃ levels rapidly. Furthermore, it is interesting to note that at 4°C and 37°C smaller amounts of Ins(1,3,4,5)P₄ and Ins(1,3,4)P₃ are formed. Whereas at 20°C, substantial amounts of Ins(1,3,4,5)P₄ and Ins(1,3,4)P₃ are formed during metabolism.

At 4°C, the reduction in these two metabolites may be attributed to the reduction in enzyme activities. However, at 37°C, this effect could be due to (a). the presence of greater Ins(1,3,4,5)P₄ 5-phosphatase activity at 37°C compared to 20°C. Thus, metabolising any Ins(1,3,4,5)P₄ formed at a greater rate, reducing the amounts of Ins(1,3,4,5)P₄ present. (b). It could be due to an increased InsP₃ 5-phosphatase activity, thus, directing the route of metabolism via Ins(1,4)P₂ and not Ins(1,3,4,5)P₄. (c). Alternatively, this could be a possible

indication of a reduced activity of InsP_3 3-kinase, thus, reducing the amount of $\text{Ins}(1,3,4,5)\text{P}$ formed or (d). an overall increase in 3-kinase activity such that $\text{Ins}(1,3,4,5)\text{P}$ is not detected after just 1 minute. All these would result in smaller amounts of $\text{Ins}(1,3,4,5)\text{P}_4$ being formed from InsP_3 , at 37°C . One approach we could adopt to establish if any of these enzymes are involved is by the use of selective enzyme inhibitors. In addition, the use of metabolic resistant analogues of inositol phosphates would reveal a temperature dependent metabolic effect.

The metabolic profile at 37°C is of considerable consequence, since the body operates at 37°C . If metabolism does occur with this profile *in vivo*, then metabolites such as $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4)\text{P}_3$ will play an insignificant role in signalling and Ca^{2+} -mobilisation. This then invokes the possible roles $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4)\text{P}_3$ may have *in vivo*. It is possible that under normal conditions, these metabolites do not play a role in cellular signalling, and are only of any significance in disease states where an overproduction of these metabolites occurs, see later.

For any subsequent experiment to investigate metabolism, platelets were maintained at 20°C as this shows the widest range of metabolites over the 10 minute period.

Pretreatment of permeabilised platelets with two inositol phosphate analogues, L- $\text{Ins}(1,4,5)\text{PS}_3$ and $\text{Ins}(1,3,5)\text{PS}_3$, produced a dose dependent inhibition of $[\text{H}^3]\text{InsP}_3$ metabolism with a concomitant potentiation of InsP_3 -induced ^{45}Ca -release.

Preincubation with L- $\text{Ins}(1,4,5)\text{PS}_3$ (3- $30\mu\text{M}$) produced a high degree of conservation of InsP_3 , a reduction in the amount of InsP_2 formed, an increase in

the amount of $\text{Ins}(1,3,4,5)\text{P}_4$ formed and a reduction in $\text{Ins}(1,3,4)\text{P}_3$ accumulation. In contrast, $\text{Ins}(1,3,5)\text{PS}_3$ ($3\text{--}30\mu\text{M}$) did not affect InsP_3 levels, however, a reduction in InsP_2 accumulation, an increase in $\text{Ins}(1,3,4,5)\text{P}_4$ formation and reduction in $\text{Ins}(1,3,4)\text{P}_3$ production was observed.

It is apparent from these studies that the two analogues, $\text{L-Ins}(1,4,5)\text{PS}_3$ and $\text{Ins}(1,3,5)\text{PS}_3$, are inhibitors of InsP_3 5-phosphatase, as indicated by the reduction in InsP_2 formation with both analogues. In addition, it appears that $\text{L-Ins}(1,4,5)\text{PS}_3$ also inhibits $\text{Ins}(1,3,4)\text{P}_3$ formation (acting on and inhibiting $\text{Ins}(1,3,4,5)_4$ 5-phosphatase) whereas $\text{Ins}(1,3,5)\text{PS}_3$ does not. This would confirm the findings of Majerus *et al.*, (Connolly *et al.*, 1985; Connolly *et al.* 1986; Majerus *et al.*, 1991; Majerus, 1992), that in platelets two 5-phosphatase enzymes are present. One responsible for the dephosphorylation of InsP_3 and the second for $\text{Ins}(1,3,4,5)\text{P}_3$ dephosphorylation.

$\text{L-Ins}(1,4,5)\text{PS}_3$ also appears to inhibit 3-kinase. As a result of the inhibition of InsP_3 5-phosphatase, metabolism will be enhanced via the InsP_3 3-kinase route and an increased formation of $\text{Ins}(1,3,4,5)\text{P}_4$ is expected. However, with $\text{L-Ins}(1,4,5)\text{PS}_3$, $30\mu\text{M}$ preincubation gives the same amount of $\text{Ins}(1,3,4,5)\text{P}_4$ as control indicating that metabolism via this route is minimal and inhibited. In addition, levels of InsP_3 are maintained higher than control suggesting that metabolism of InsP_3 by both InsP_3 5-phosphatase and 3-kinase is inhibited.

In the presence of $\text{Ins}(1,3,5)\text{PS}_3$, increased formation of $\text{Ins}(1,3,4,5)\text{P}_4$ suggests that InsP_3 3-kinase is functional and not affected. $\text{Ins}(1,3,5)\text{PS}_3$ is not a substrate for 3-kinase, probably due to the presence of a phosphorothioate group on the 3-position. Making it a selective, non- Ca^{2+} mobilising inhibitor of InsP_3 5-phosphatase.

The phosphorothioate analogue of InsP_3 , $\text{Ins}(1,4,5)\text{PS}_3$, was found to be a potent inhibitor of InsP_3 5-phosphatase. Because it is known that the D-isomers of InsP_3 analogues are substrates for 5-phosphatase, and that L- InsP is a weak inhibitor of 5-phosphatase, it was thought that the phosphorothioate analogue of L- $\text{Ins}(1,4,5)\text{P}_3$ would prove to be a more potent inhibitor than the parent compound. Thus, L- $\text{Ins}(1,4,5)\text{PS}_3$ was originally synthesized as 5-phosphatase inhibitor. A clear picture has emerged from our work to confirm this hypothesis, and L- $\text{Ins}(1,4,5)\text{PS}_3$ was found to inhibit both InsP_3 5-phosphatase and $\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase.

The novel analogues investigated in this study thus provide new tools with which InsP_3 metabolism and function can be modulated, as they are potent InsP_3 5-phosphatase inhibitors. Due to the lack of specific InsP_3 receptor antagonists, specific enzyme inhibitors may help elucidate the functional significance of InsP_3 metabolism in signalling. Recently, reports indicate that disulfiram and its analogues inhibit InsP_3 5-phosphatase at micromolar concentrations (Fowler *et al.*, 1993). However, these compounds were found toxic to the cell and reduced cell proliferation significantly, and so can not be used to *in vivo*. In addition, a calmodulin inhibitor calmidazolium chloride appeared to inhibit the 5-phosphatase with an IC_{50} of $100\mu\text{M}$, however its actions were thought not to be through calmodulin inhibition (Fowler and Eriksson, 1993). Furthermore, Li^+ which is widely used in the treatment of affective disorders is thought to exert its actions through inhibiting the two enzymes, inositol 1-phosphatase and inositol monophosphatase thus disrupting the signalling mechanism.

A potentially important recent development is the recognition of a second dephosphorylation pathway of InsP_3 to *myo*-inositol 4,5-bisphosphate [$\text{Ins}(4,5)\text{P}_2$] in the presence of Li^+ in neural tissue (Hughes and Michell, 1993)

and receptor-stimulated accumulation of $\text{Ins}(4,5)\text{P}_2$ in the presence of Li^+ was demonstrated. The significance of formation of $\text{Ins}(4,5)\text{P}_2$ is not yet known. $\text{Ins}(4,5)\text{P}_2$ can release Ca^{2+} from intracellular stores with 100 fold less potency than InsP_3 . In addition, $\text{Ins}(4,5)\text{P}_2$ was shown to inhibit the calmodulin-dependent Ca^{2+} -ATPase pump of erythrocytes (Hughes and Michell, 1993). A recent finding suggests that beryllium ions competitively inhibit inositol monophosphatase, being three times more potent than Li^+ (Faraci *et al.*, 1993). All these agents will help develop a clearer understanding of PI signalling.

The significance of defects in inositol phosphate metabolism at the 5-phosphatase enzyme is depicted in diseases such as Lowe's oculocerebrorenal syndrome (OCRL) where an inborn error in inositol phosphate metabolism has been implicated (Attree *et al.*, 1992). Furthermore, HIV-infected lymphoblastoid cells show abnormal metabolism with chronically elevated levels of $\text{Ins}(1,3,4,5)\text{P}_4$ (Nye *et al.*, 1992) and there is evidence that inositol monophosphatase activity is elevated in schizophrenic patients (Hughes and Michell, 1993). It is clear that selective 5-phosphatase inhibitors are required to investigate inositol phosphate metabolism and the putative role of $\text{Ins}(1,3,4,5)\text{P}_4$ and so far the two analogues investigated provide the best leads for further syntheses of more selective and potent enzyme inhibitors. A crucial need, however, is to make these inhibitors membrane permeable.

Purified platelet InsP_3 3-kinase was shown to contain two polypeptides (53 and 36 kDa) both of which expressed activity (Lin *et al.*, 1993) and the catalytic site was shown to be located at the C-terminal end of the protein. However, other data indicate that the two polypeptides are indeed two isozymes of InsP_3 3-kinase (Lin *et al.*, 1993). While the analogues investigated in this study did not suggest the presence of isozymes, nevertheless the

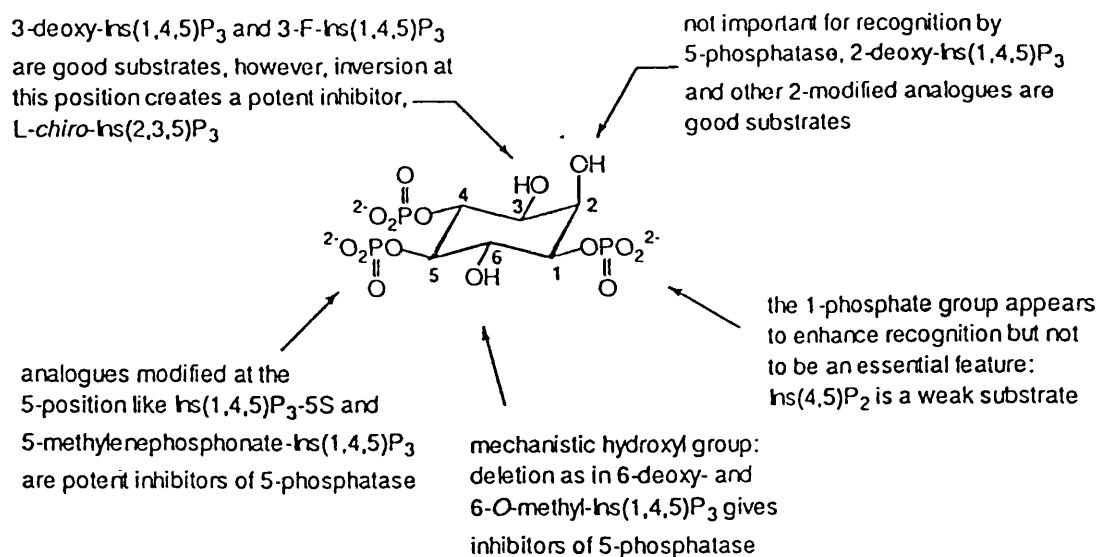


Figure 71. Summary of the requirements of the 5-phosphatase

analogues may be nonspecific 3-kinase inhibitors thus showing no selectivity for the different isozymes. Still, considering that the purified InsP_3 3-kinase has a higher affinity for InsP_3 (K_m $0.76\mu\text{M}$) than that of the platelet InsP_3 5-phosphatase (K_m $25\mu\text{M}$) (Mitchell *et al.*, 1989), the purification of this enzyme should facilitate further investigations concerning the possible role this enzyme plays in platelet function. The functional significance of InsP_3 3-kinase has been studied and suggestions that InsP_3 3-kinase may act as a regulator of the Ca^{2+} -phosphoinositide signal transduction mechanism (Balla *et al.*, 1991) have been proposed. It was found that in fibroblasts transfected with rat brain 3-kinase, the conversion of InsP_3 to $\text{Ins}(1,3,4,5)\text{P}_4$ is greatly increased, with a concomitant reduction in InsP_3 levels and the attenuation of the Ca^{2+} response (mobilisation and influx).

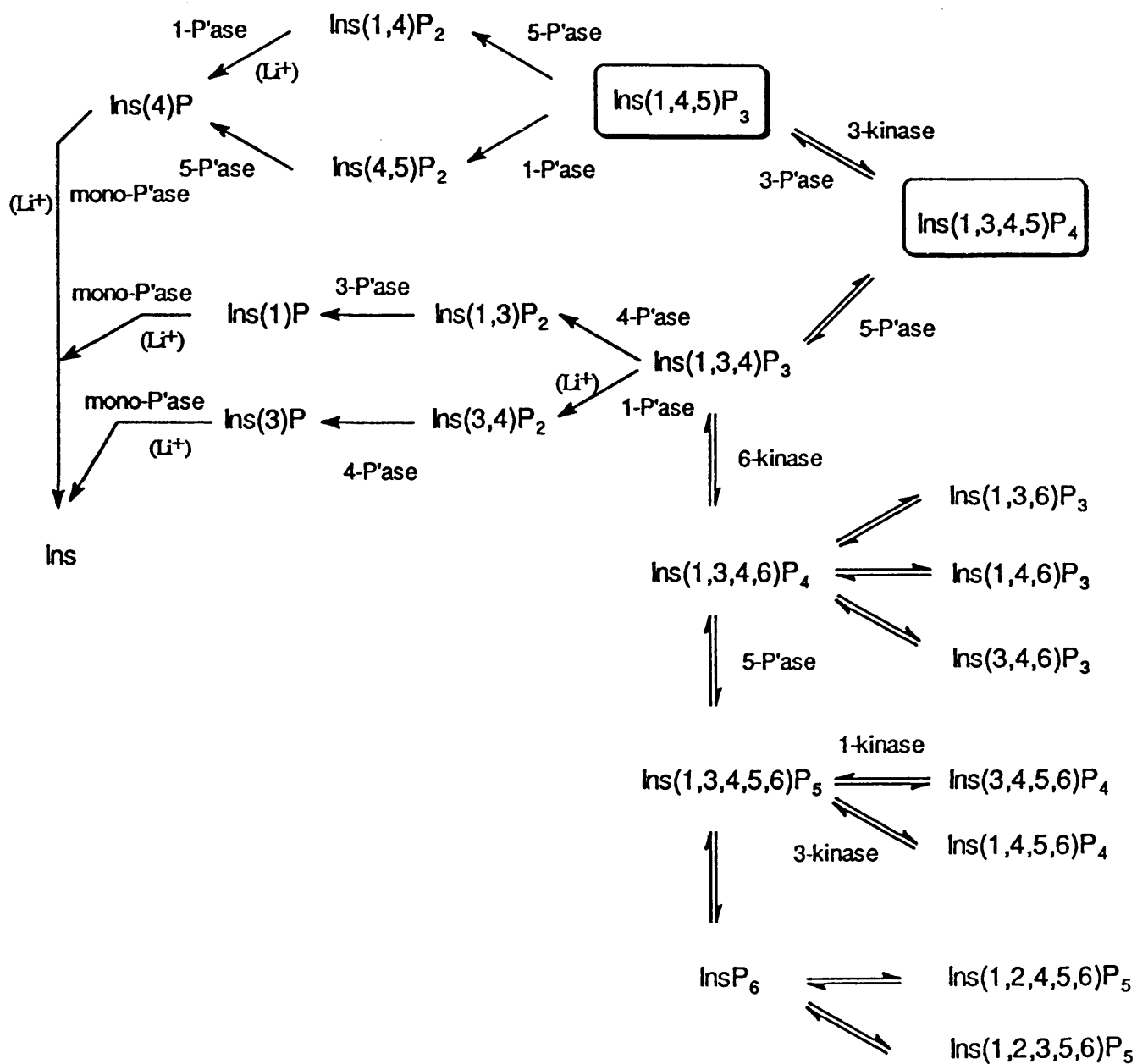


Figure 72. InsP_3 and its metabolites. An up to date version of InsP_3 metabolism and the various metabolites formed along the way taken from Hughes and Michell (1993).

4.3 Ca²⁺ entry and the effect of SKF 96365 in platelet signalling.

Cytosolic calcium elevation can occur by one of three ways; (i) the release from intracellular stores upon PIP₂ breakdown and InsP₃ and DAG production (Berridge, 1993); (ii) through voltage operated Ca²⁺ channels in which the developments of drugs that act as antagonists such as dihydropyridines and phenylalkylamines at the receptor site has greatly contributed to understanding this area ; (iii) through receptor mediated Ca²⁺ entry (RMCE) of which little was understood until the recent development of two imidazol derivatives [(+)-1-2,3-bis(4-methoxyphenyl)-methoxy)propyl]1-H-imidazol (SC 38249) and its 4-methoxyphenyl derivative SKF 96365, which have provided tools for the study of RMCE. SKF 96365 has been shown to inhibit Ca²⁺ influx having no effect on mobilisation in a variety of cells including platelets, neutrophils and endothelial cells (Merritt *et al.*, 1990*ab*; Ciardo and Meldolesi, 1990; Chan and Greenberg, 1991).

In this study, using fura-loaded platelets, racemic SKF 96365, whose structure can be seen in figure 73, was found to dose-dependently inhibit PAF-induced Ca²⁺ release. In order to establish if inhibition is at the receptor or post-receptor level, the effect of SKF 96365 on InsP₃-production upon PAF stimulation was measured and SKF 96365 was found to dose dependently inhibit InsP₃-formation with a similar EC₅₀ to the inhibition of PAF-induced Ca²⁺ release in fura-loaded platelets. This suggests, contrary to the literature, that SKF 96365 is inhibiting Ca²⁺ release through the inhibition of InsP₃ formation. However, the contributions of Ca²⁺-influx and Ca²⁺-mobilisation to the Ca²⁺-release detected were not determined and it may be that InsP₃-induced Ca²⁺-mobilisation plays a minor insignificant role in the Ca²⁺-response observed here. Thus, InsP₃ having a minor role, and that SKF 96365 is still exerting its effect through a major

inhibition of Ca^{2+} influx. This situation is further complicated by the finding that SKF 96365 causes a dose dependent inhibition of dense granule release at concentrations an order of magnitude lower than that observed with the inhibition of Ca^{2+} -release and InsP_3 formation. It is possible that dense granule release is more sensitive to the other product of PIP_2 hydrolysis, namely DAG, and inhibiting DAG formation may inhibit the platelet release reaction. Further, it is conceivable that SKF 96365 may have an intrinsic effect on some aspect of platelet function, by inhibiting adhesion, shape change or aggregation. Thus, preventing dense granule release.

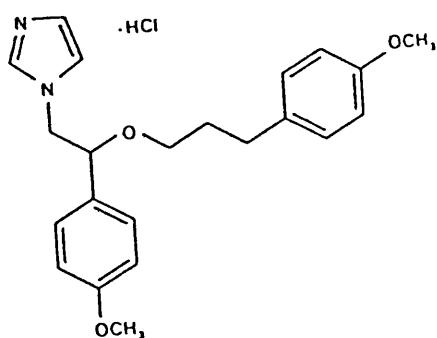


Figure 73. The structure of SKF 96365

SKF 96365 did not mobilise Ca^{2+} from permeabilised platelets. In addition it did not effect InsP_3 - or $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Ca^{2+} release indicating that SKF 96365 does not possess the structural requirements for receptor binding.

These findings are consistent with our previous results where SKF 96365 was found to inhibit Ca^{2+} release through inhibiting InsP_3 -production, possibly through inhibiting PIP_2 hydrolysis. Merritt *et al* (1990*ab*) suggest in their initial studies that SKF 96365 can interfere with mobilisation of Ca^{2+} from intracellular stores, and Chan & Greenberg (1991) find that elevations in $[\text{Ca}^{2+}]_i$

are also inhibited with SKF 96365. However both of these groups report that this is a negligible effect not mediated through InsP_3 .

The Ca^{2+} -influx inhibitor in our system was not found to be selective in blocking Ca^{2+} -release through RMCC, rather it was found to inhibit Ca^{2+} release induced by PAF and further investigations indicate that this effect is through inhibiting InsP_3 formation.

The indirect control InsP_3 has on Ca^{2+} entry is illustrated in the capacitative Ca^{2+} entry hypothesis. This is based on the finding that emptying intracellular Ca^{2+} stores is itself sufficient to activate Ca^{2+} entry (Irvine, 1992) based on the results using thapsigargin, an inhibitor of Ca^{2+} -ATPase (Takemura *et al.*, 1991). However, as a result of this hypothesis, a major question is left unanswered, and this is : How does the Ca^{2+} content of the intracellular stores communicate with the plasma membrane so as to control Ca^{2+} entry ?.

Principally three mechanisms have been proposed;

(i) The low luminal Ca^{2+} levels may stimulate the generation of a soluble second messenger that acts upon a receptor in the plasma membrane. Recent reports have isolated such a molecule which caused Ca^{2+} influx when applied to cells and was named CIF (Ca^{2+} -influx factor) (Randriamampita and Tsien, 1993), further, Parekh *et al.*, (1993) have also found the presence of a small diffusable messenger and they suggest that the molecule is not an inositol phosphate.

(ii) Low luminal Ca^{2+} may result in low free Ca^{2+} near the plasma membrane (Putney, 1986), and evidence shows that decreasing cytosolic Ca^{2+} can affect Ca^{2+} entry (Irvine, 1992). However, in experiments where $[\text{Ca}^{2+}]_i$ is raised by

agonists such as thapsigargin, Ca^{2+} entry is still initiated despite the free Ca^{2+} near the plasma membrane being high (Irvine, 1992).

(iii) Communication could occur via a protein (or proteins) anchored in the ER such that its structure is modulated by luminal Ca^{2+} levels (Irvine, 1992). Other reports have proposed that InsP_3 may be directly involved in the control of Ca^{2+} entry. Intracellular applications of InsP_3 to sea urchin eggs, lacrimal gland cells and mast cells produce a response indicative of activation of both Ca^{2+} mobilisation and Ca^{2+} entry from the extracellular space (Putney, 1992). In addition Heparin, an InsP_3 receptor antagonist, blocks both phases of the $[\text{Ca}^{2+}]_i$ signal. However, in some cases it appears that the presence of $\text{Ins}(1,3,4,5)\text{P}_4$ is necessary for the second Ca^{2+} entry phase. In most studies, InsP_3 applied directly to the plasma membrane does not increase their Ca^{2+} permeability. Furthermore, InsP_3 receptors have not been revealed on the plasma membrane of Purkinje cells. (Putney, 1992).

$\text{Ins}(1,3,4,5)\text{P}_4$ is rapidly formed whenever InsP_3 is produced in a cell, and whether or not it can control Ca^{2+} entry by interacting with specific receptors (see figure 74) is still controversial (Irvine, 1992). Irvine and colleagues were the first to propose a role for $\text{Ins}(1,3,4,5)\text{P}_4$ in the control of Ca^{2+} entry (Irvine *et al.*, 1985; Irvine, 1988; Irvine, 1991). However, Bird *et al.*, (1991) opposed this idea where they found that in mouse lacrimal cells InsP_3 without the presence of $\text{Ins}(1,3,4,5)\text{P}_4$ caused Ca^{2+} entry, and they argued that in previous experiments sufficient InsP_3 was not added to see this effect and in those cases $\text{Ins}(1,3,4,5)\text{P}_4$ was acting to protect InsP_3 from metabolism (Berridge and Irvine, 1989) and itself was being converted back to InsP_3 via a 3-phosphatase (Hoer *et al.*, 1988; Kjeldsen *et al.*, 1988; Shears, 1989; Bird *et al.*, 1991).

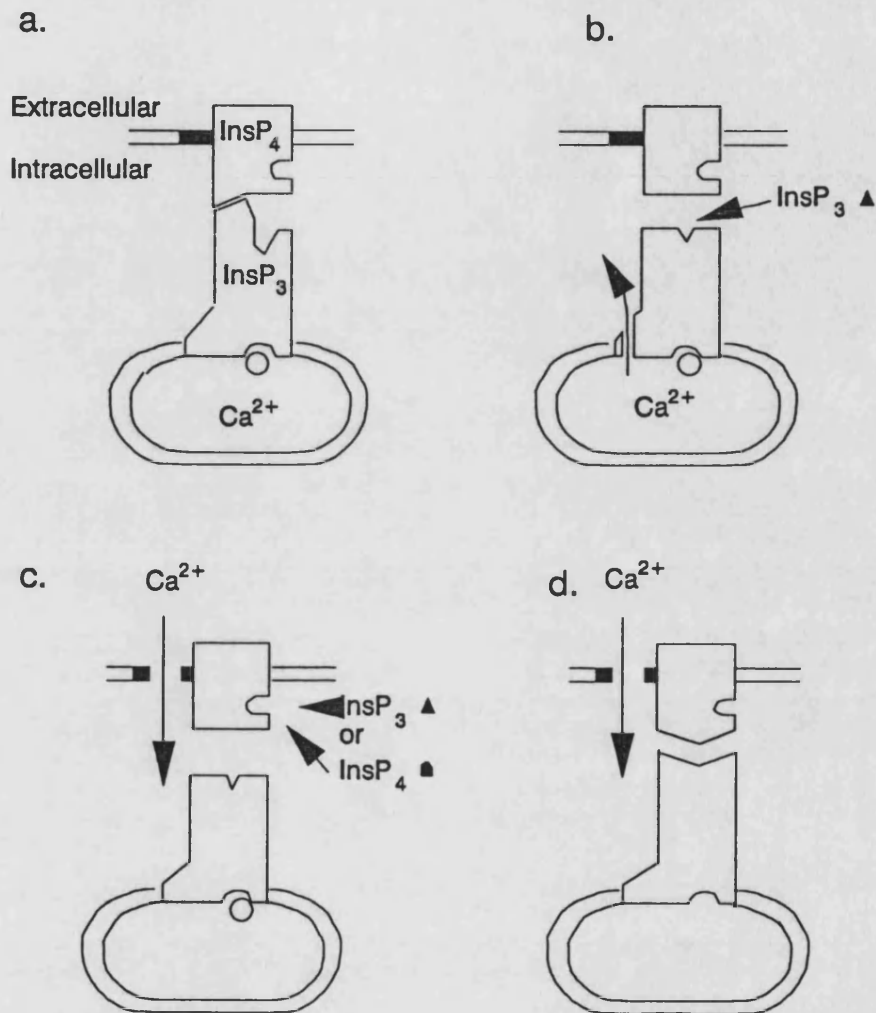


Figure 74. The involvement of Ins(1,3,4,5)P_4 in Ca^{2+} entry. The interaction between the InsP_3 receptor and the Ins(1,3,4,5)P_4 receptor. a. resting cell; b. InsP_3 production and binding to its receptor causes Ca^{2+} -mobilisation and leads to a conformational change in the Ins(1,3,4,5)P_4 receptor; c. Ins(1,3,4,5)P_4 or InsP_3 binding to the Ins(1,3,4,5)P_4 receptor leads to; d. Ca^{2+} -entry.

Further studies by Smith *et al.*, (1992) showed that although very high levels of InsP₃ do cause Ca²⁺ entry, Ins(1,3,4,5)P₄ additions could further enhance Ca²⁺ entry.

Luckhoff and Clapham in their work give a direct demonstration of activation by Ins(1,3,4,5)P₄ of a Ca²⁺-channel in endothelial cells (Luckhoff and Clapham, 1992). This effect is reversible and not mimicked by InsP₃, except at high doses and InsP₃ does not enhance this effect. However, Luckhoff and Clapham (1992) used a relatively high free Ca²⁺ (0.5μM) in order to see their channel, and at these concentrations, the Ca²⁺-channel is already partially activated, and this activation was further increased by increasing [Ca²⁺] up to 1mM.

Thus, although there is considerable evidence for the role of inositol phosphates in Ca²⁺ entry, the nature of this role and the specific inositol phosphates involved is much less clear. This is likely due, at least in part, to difficulties in assaying the effects of inositol phosphates on Ca²⁺ entry. Investigations into the effects of inositol phosphates on Ca²⁺ entry require methods for introducing these highly polar hydrophilic compound into the cells, while maintaining sufficient support of the plasma membrane to permit measurements of its permeability to Ca²⁺. The techniques most used are patch-clamp, both in whole cell and excised patch configurations and microinjection. Much of the confusion in the field of receptor-regulated Ca²⁺ entry is generated by reports of conflicting findings derived from studies with these different approaches, which may be in part attributable to the difficulties and problems inherent to each approach.

It is beyond any doubt that Ins(1,3,4,5)P₄ can do something. It binds to a receptor protein (Donié and Reiser, 1991; Cullen and Irvine, 1992), synergizes with InsP₃ to mobilise Ca²⁺ (Joseph *et al.*, 1987; Irvine and Moor, 1987), can effect Ca²⁺ reuptake (Boynton *et al.*, 1990) or cause Ca²⁺ entry (Guse *et al.*, 1991) for review see (Irvine, 1992; Irvine, 1991). However, experimental artifacts are being generated which hamper our understanding of the role of this molecule and the advent of inositol phosphate analogues that are capable of crossing cell membranes, such as lipophilic analogues, will no doubt help overcome the problems associated with accessibility of the compounds to the intracellular compartment without having to disrupt the plasma membrane. The variation in results regarding Ins(1,3,4,5)P₄ studies can also be overcome with the use of phosphorothioate analogue of Ins(1,3,4,5)P₄, namely Ins(1,3,4,5)PS₄ and Ins(3)PS(1,4,5)P₃. Ins(1,3,4,5)PS₄ is predicted to be resistant to 5-phosphatase metabolism and would help elucidate the ambiguous role played by Ins(1,3,4,5)P₄ in Ca²⁺ entry.

4.4 The effects of InsP₃ and analogues on Ca²⁺ release and the functional responses; dense granule release and aggregation.

4.4.1 Fluorescent studies on permeabilised platelets.

Since the advent of fluorescent dyes such as quin-2, fura-2 and fluo-3, they have been used extensively to measure Ca²⁺-release induced by InsP₃ in a wide variety of cell membrane and permeabilised cell preparations (Guillemette and Segui, 1988; Debs *et al.*, 1988; Champeil *et al.*, 1989; Mohr *et al.*, 1993). However, to date studies using fluorescent dyes and permeabilised platelets or platelet preparation have not been used to assess InsP₃-induced release of Ca²⁺.

In this study we set out to develop a method for the use of fluorescent dyes for the detection of Ca^{2+} release in permeabilised platelets and two platelet membrane preparations, a crude and pure preparation, and both the acetoxymethyl ester and the free acid of fura-2 have been used in this study. In all cases, regardless of buffer, permeabilisation technique (electrical, saponin, streptolysin-O), the use of permeabilised platelets or membranes, the use of different Ca^{2+} chelators (EGTA, EDTA, BAPTA) or simply passing the buffer or source of water over a Ca^{2+} -chelating agent such as chelex or Ca^{2+} -sponge, InsP_3 -induced Ca^{2+} -release was not detected (data not shown). Due to the low intracellular $[\text{Ca}^{2+}] \approx 100\text{nM}$ and the high extracellular $[\text{Ca}^{2+}] \approx 1\text{mM}$ it is essential to reduce the Ca^{2+} content of the platelet suspension buffer to avoid platelet activation upon permeabilisation. Further buffering is necessary in order to detect small $[\text{Ca}^{2+}]_i$ elevations above basal upon agonist stimulation, where if basal $[\text{Ca}^{2+}]_i$ was $1\mu\text{M}$, a 100nM rise in $[\text{Ca}^{2+}]$ would not be easily detected.

In this study an appropriate buffering system could not be established on account of the sensitivity of the system to $[\text{Ca}^{2+}]$. Overbuffering with agents like EGTA and EDTA leads to the quenching of any signal produced upon InsP_3 activation. On the other hand underbuffering leads to high $[\text{Ca}^{2+}]$ present upon permeabilisation, which will mask any elevation in $[\text{Ca}^{2+}]$ upon InsP_3 addition. Thus an alternative method for reducing $[\text{Ca}^{2+}]$ was investigated. Here the platelet buffer was passed over a Ca^{2+} chelators such as chelex or a Ca^{2+} -sponge in order to reduce the Ca^{2+} content. This was not found to have any additional benefits over the previous Ca^{2+} -chelators used, and Ca^{2+} release upon InsP_3 -addition could not be detected (data not shown).

Various reports suggest leaving $[\text{Ca}^{2+}]$ unbuffered, and after permeabilisation the buffer is supplemented with ATP which will initiate Ca^{2+} -uptake via the

Ca²⁺-ATPase pump (Renard *et al.*, 1992; Combettes *et al.*, 1993; Hofer and Machen, 1993). However, in this study ATP did not initiate Ca²⁺ uptake, and the platelets became fully activated upon permeabilisation. In addition, in most cases ATP added to the cuvette was found to quench the fluorescence signal. Other reports do not modify [Ca²⁺] (Mohr *et al.*, 1993; Hershey *et al.*, 1993). In addition, ATP and Mg²⁺ are excluded from the buffer as a result of the concern that ATP will inhibit InsP₃ binding to its receptor.

Furthermore, a recent report (Richardson and Taylor, 1993) suggests that Ca²⁺ chelators such as EGTA, EDTA and BAPTA act to inhibit InsP₃-stimulated Ca²⁺ release because they will act as competitive antagonists of InsP₃ binding to its receptor. These Ca²⁺ chelators are often used to buffer the free [Ca²⁺] of the various buffers in which the cells are suspended. Thus, these chelators will interfere with InsP₃ binding to its receptor and any subsequent Ca²⁺ release or functional effect to be studied.

To date, there are little data available using fluorescent Ca²⁺ studies in permeabilised platelets and in the light of this work, we will not be able to contribute to this deficiency.

4.4.2 Functional studies; aggregation and dense granule release.

It is widely accepted that in most cells InsP₃ mobilises Ca²⁺ which leads to various functional responses and the same is true of platelets. InsP₃ has been shown to induce aggregation (Knezevic *et al.*, 1992) and dense granule secretion (Brass and Joseph, 1985) from permeabilised platelets and our aim in this study was to set up such methods in order to assess the effect of InsP₃

analogues on platelet function and to establish if a correlation between InsP_3 -induced Ca^{2+} release and platelet functional responses exists.

Upon saponin permeabilisation of platelets, an aggregatory response was observed (data not shown). Although a previous report by Knezevic *et al.*, (1992) suggest that there exists a narrow saponin concentration range which facilitates InsP_3 -mediated responses, without leading to platelet activation, as shown in permeabilised platelets supplemented with $100\mu\text{g/ml}$ fibrinogen, this was not apparent in our study. An all or nothing effect was observed with saponin in our system where saponin either produced aggregation and permeabilisation at $5.25\mu\text{g/ml}$ or not at concentrations of $5\mu\text{g/ml}$, where permeabilisation was not achieved as indicated by the lack of aggregation upon 1mM Ca^{2+} addition. Modifications to the suspension buffer in terms of Ca^{2+} buffering, salt constituents and a different permeabilising chemical did not cause permeabilisation without aggregation (data not shown).

Thus, aggregation could not be used to investigate the functional effects of inositol phosphate analogues due to the inherent aggregation produced upon permeabilisation.

InsP_3 -induced dense granule release was also investigated using permeabilised platelets. Brass & Joseph (1985) use an EGTA buffered system to demonstrate that Ca^{2+} alone causes $[^{14}\text{C}]5\text{-HT}$ release and addition of InsP_3 shifted this curve to the left where InsP_3 causes an additional $[^{14}\text{C}]5\text{-HT}$ release, in our system, this separation between Ca^{2+} and InsP_3 induced dense granule release was also present ($\approx 10\text{-}15\%$ of total $[^{14}\text{C}]5\text{-HT}$ release). However, this window was too small for any further studies such as dose response curves to InsP_3 and analogues.

InsP₃ being hydrophilic requires permeabilised cell systems, in order to gain access to the intracellular compartment and although permeabilised cells are more physiological than membrane preparation, it is desirable to study these compounds in an intact cell system. Knight & Scrutton (1987) have used a high electric discharge to render platelets permeable. Furthermore, incubating cells at 37°C for 60 minutes reseals electroporated cells (Hughes and Crawford, 1990). This technique was used to encapsulate hydrophilic InsP₃ or analogues for the later investigation on Ca²⁺ release, aggregation and dense granule release. Due to the small size of the platelet, a high electric field of 10- 20kV is necessary (Knight and Scrutton, 1987; Hughes and Crawford, 1990; Daniel *et al.*, 1992) with 10 cycles. This high voltage however could not be achieved by the electroporator available for this study, 5kV being the maximum attainable. Hence, the number of cycles was increased to compensate for this shortcoming. It was observed that upon electroporation, platelets did become permeable (as indicated by dense granule release due to Ca²⁺ addition). However, upon incubating at 37°C for 60 minutes, all responsiveness of the platelets is lost.

A further complication is that platelet functional responses are optimal at 37°C. However, as demonstrated by the metabolism data in this thesis, InsP₃ is very rapidly metabolised at this temperature and this may hamper those studies where the local InsP₃ concentration at the receptor may be greatly reduced due to metabolism.

We attempted to set up a functional assay in platelets in order to assess the effects of InsP₃ and its analogues, especially the partial agonists and the 5-phosphatase enzyme inhibitors, on platelet function and to further elucidate the roles of InsP₃ in platelet activation and responses. Of interest would have been the effect of the partial agonists on function to see whether partial activation of

the platelets would occur, whether a smaller percentage of the dense granules would be released or if aggregation would be attenuated. However, when we consider the mechanisms of platelet activation, involving the many positive feedback mechanisms involved in activation and dense granule release it would appear that partial activation would not be possible. In addition, the significance of one metabolite over another in platelet activation and function could have been addressed by investigating the effect of the metabolic enzyme inhibitors, L-Ins(1,4,5)PS₃ and Ins(1,3,5)PS₃, on platelet function.

Research into PI signalling would substantially benefit from the synthesis of lipophilic analogues of InsP₃. These analogues would be capable of crossing cell membranes and the need for cell poration or membrane preparations would not be required. This would provide a more physiological environment for the study of these compounds. In addition, the cells would not be exposed to such harsh treatments such as a high electric field or detergents for poration which affect the cell's integrity and function.

4.5 Summary of the discussion.

We have been able to demonstrate stereospecific InsP₃-induced Ca²⁺-release from permeabilised platelets. D-InsP₃ is a potent full agonist with an EC₅₀ of 0.18 ± 0.01 μM at 4°C (1.14 μM at 20°C) and L-InsP₃ is completely inactive. In addition, InsP₃-induced Ca²⁺-release was shown to be temperature dependent, 4°C being the optimum temperature for release reactions probably due to the slow rate of metabolism at this temperature.

From this thesis, a structure/activity relationship has become apparent and it is possible to predict, to a limited extent, the activity of novel analogues in

permeabilised platelets from this relationship. The three phosphates at the 1-, 4- and 5-positions are essential for activity, and removal of any of these, especially the vicinal 4,5-bisphosphate, causes a substantial loss in activity. Of the hydroxyl groups, the 2- and 3-OH's do not appear to be important. However, deletion of the 6-OH causes a significant loss in activity indicating that this may be essential for receptor recognition or Ca^{2+} -mobilisation.

It appears that phosphate substitution with phosphorothioates decreases in intrinsic activity, although this is not always the case as in $\text{Ins}(1,3,4,6)\text{PS}_4$ and $\text{Ins}(1,4,5)\text{PS}_3$. In this study, three such analogues have been investigated, and these are important because it appears from their structures the requirements for InsP_3 -binding are met in that they all possess pseudo 1-, 4- and 5- phosphates. They also possess an equatorial pseudo 6-OH of InsP_3 . The partial agonist with the highest intrinsic activity, *scyllo*- $\text{Ins}(1,2,4,5)\text{PS}_4$ possesses a phosphorothioate group at the 2-position. $\text{Ins}(1,4,6)\text{PS}_3$ is a partial agonist possessing the lowest intrinsic activity. It mobilised a maximum of 20% of the sequestered ^{45}Ca , and was able to antagonise the effect of InsP_3 .

To date, a specific selective antagonist at the InsP_3 receptor is still not available. The two antagonists available, heparin and decavanadate, are large molecules, non-specific in their activity and possess other activities such as enzyme inhibition. Thus, our aim is to uncover small molecule antagonists, based on the structure of InsP_3 . From the data obtained with studies using partial agonists, we now have a better idea of the requirements for potency and intrinsic activity. We may be able to develop these to make partial agonists with even lower intrinsic activity, with the aim to develop an antagonist.

Studies using partial agonists indicate the possibility of tissue-specific InsP_3 receptors. $\text{Ins}(1,3,4,6)\text{P}_4$ and 6-deoxy- $\text{Ins}(1,4,5)\text{P}_3$ were found to be partial

agonists in SH-SY5Y neuroblastoma cells (Gawler *et al.*, 1991; Safrany *et al* 1993) but were found to be full agonists in permeabilised platelets (this thesis). In addition, some analogues appear to be full agonists with low potency in permeabilised platelets, e.g. D/L-Ins(1,3,4)P₃ has an EC₅₀ of $\geq 30\mu\text{M}$ in permeabilised platelets. The Ca²⁺-release profile by D/L-Ins(1,3,4)P₃ is probably due to the presence of the L-isomer, the D-isomer being completely inactive. Strupish *et al.*, (1988) found that D-Ins(1,3,4)P₃ was presumably inactive in Swiss 3T3 cells and in permeabilised smooth muscle cells with an EC₅₀ of 120 μM (Polokoff *et al.*, 1988). The findings that the same analogues possess different activities in different tissues points towards the possibility of tissue specific receptors.

The calcium influx inhibitor, SKF 96365, was used to probe the role of Ca²⁺ entry in InsP₃ signalling and to probe the contentious issue of the synergistic actions of InsP₃ and Ins(1,3,4,5)P₄ acting to elevate intracellular Ca²⁺ with InsP₃ mobilising intracellular Ca²⁺ and Ins(1,3,4,5)P₄ facilitating Ca²⁺ entry. However, in our hands SKF 96365 was found not to be a pure Ca²⁺-influx inhibitor, but it appeared to have an effect on PI signalling. It was capable of inhibiting PAF-induced InsP₃-production, Ca²⁺-release and [¹⁴C]5-HT release which is a result of InsP₃-production and the subsequent Ca²⁺-elevation. Consequently, we were unable to make as effective a use of this compound as we had initially hoped.

Platelets and platelet activation play an important role in coronary disease and thrombosis. Thus, modulating platelet function may have a role in controlling these diseases. Blocking platelet activation could be achieved by blocking receptor activation by the use of specific receptor antagonists. However, this is not feasible when considering the number of intrinsic molecules and agents

released by platelets and other immune cells that act to activate platelets and a large number of specific platelet antagonists would be required.

Alternatively, activation can be prevented by blocking the signalling mechanisms involved in platelet activation. Since most platelet agonists signal through PI, elevating InsP_3 levels and subsequently Ca^{2+} , modulating this aspect of platelet activation is a more viable proposition. Data from this thesis indicates the possibility of tissue specific receptors. Thus, uncovering a specific InsP_3 receptor antagonist for platelets would present a real possibility of modulating platelet activation and may have long term implications in diseases such as thrombosis. This in conjunction with the subsequent synthesis of lipophilic analogues of InsP_3 receptor antagonists would have great therapeutic pertinence.

APPENDIX I:

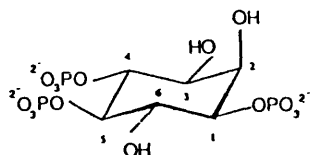
Names and structures of inositol phosphate analogues examined.

No.	Compound & Structure	EC ₅₀ and activity
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FA = full agonist

PA = partial agonist.

1.



$0.18 \pm 0.1 \mu\text{M}$ (4°C) FA

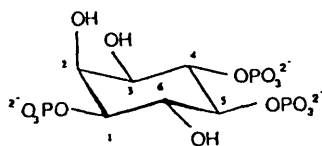
$1.14 \pm 0.2 \mu\text{M}$ (20°C) "

$\geq 10 \mu\text{M}$ (37°C) "

D-*myo*-inositol 1,4,5-trisphosphate

D-Ins(1,4,5)P₃

2.

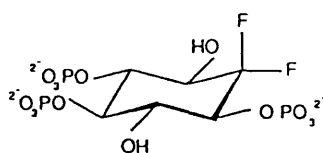


$\geq 100 \mu\text{M}$ (20°C) Inactive

L-*myo*-inositol 1,4,5-trisphosphate

L-Ins(1,4,5)P₃

3.

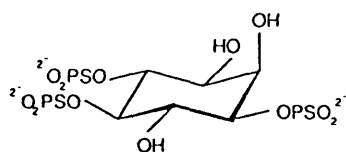


$0.63 \pm 0.09 \mu\text{M}$ (20°C) FA

D-*myo*-inositol 2,2-difluoro 1,4,5-trisphosphate

Ins(2,2)F₂(1,4,5)P₃

4.

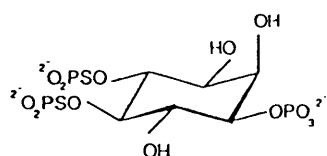


$0.8 \pm 0.1 \mu\text{M}$ (20°C) FA

D-*myo*-inositol 1,4,5-trisphosphorothioate

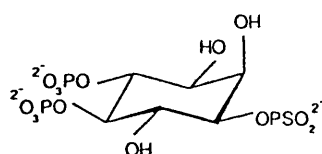
Ins(1,4,5)PS₃

5.


 $0.85 \pm 0.1 \mu\text{M}$ (20°C) FA

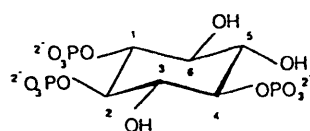
D-*myo*-inositol-1-phosphate 4,5-
bisphosphorothioate
Ins(1)P(4,5)PS₂

6.


 $0.65 \pm 0.1 \mu\text{M}$ (20°C) FA

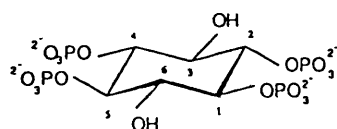
D/L-*myo*-inositol-1-phosphorothioate
4,5-bisphosphate
Ins(1)PS(4,5)P₂

7.


 $0.58 \pm 0.07 \mu\text{M}$ (20°C) FA

scyllo-inositol 1,2,4-trisphosphate
scyllo-Ins(1,2,4)P₃

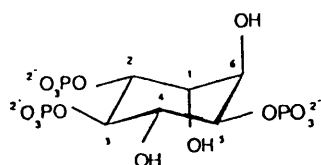
8.


 $0.1 \pm 0.05 \mu\text{M}$ (4° & 20°) FA

 $5.1 \pm 0.2 \mu\text{M}$ (37°C) "

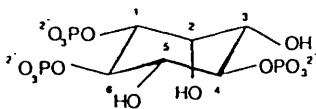
scyllo-inositol 1,2,4,5-tetrakisphosphate
scyllo-Ins(1,2,4,5)P₄

9.

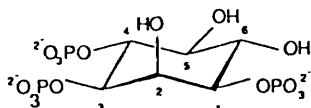

 $1.9 \pm 0.1 \mu\text{M}$ (4°C) FA

L-*chiro* 2,3,5-trisphosphate
L-*chiro*-Ins(2,3,5)P₃

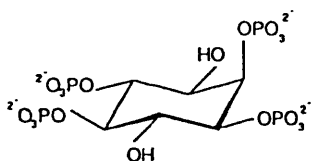
10.

 $2.07 \pm 0.08 \mu\text{M}$ (4°C) FAD-*myo*-inositol 1,4,6-trisphosphateIns(1,4,6)P₃

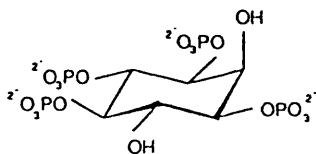
11.

 $\geq 10 \mu\text{M}$ (4°C) FAL-*myo*-inositol 1,3,4-trisphosphateL-Ins(1,3,4)P₃

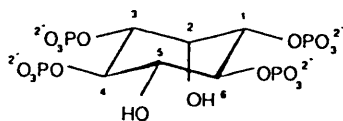
12.

 $0.53 \pm 0.04 \mu\text{M}$ (20°C) FAD-*myo*-inositol 1,2,4,5-tetrakisphosphateIns(1,2,4,5)P₄

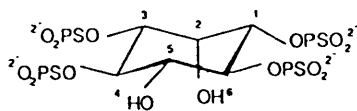
13.

 $\geq 10 \mu\text{M}$ (20°C) FAD-*myo*-inositol 1,3,4,5-tetrakisphosphateIns(1,3,4,5)P₄

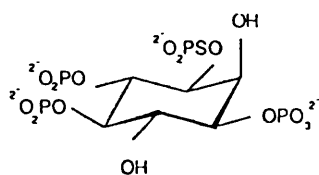
14.

 $9.67 \pm 0.5 \mu\text{M}$ (20°C) FAD-*myo*-inositol 1,3,4,6-tetrakisphosphateIns(1,3,4,6)P₄

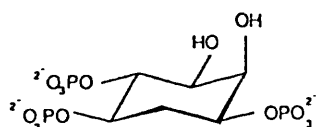
15.

 $\geq 10\mu\text{M}$ (4°C) FAD-*myo*-inositol 1,3,4,6-tetrakisphosphorothioateIns(1,3,4,6)PS₄

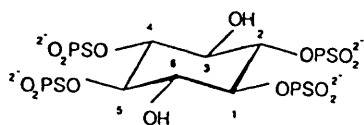
16.

 $\geq 10\mu\text{M}$ (4°C) FAD-*myo*-inositol 3-phosphorothioate 1,4,5-trisphosphateIns(3)PS(1,4,5)P₃

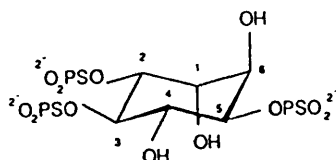
17.

 $\geq 30\mu\text{M}$ (20°C) FAD-*myo*-inositol-6-deoxy 1,4,5-trisphosphate6-deoxy-Ins(1,4,5)P₃

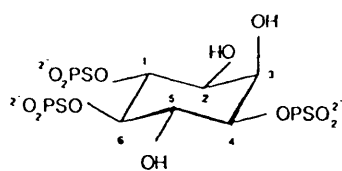
18.

 $\geq 30\mu\text{M}$ (20°C) PA*scyllo*-inositol 1,2,4,5-tetrakisphosphorothioate*scyllo*-Ins(1,2,4,5)PS₄

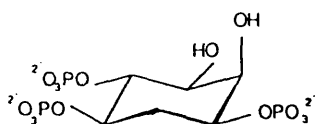
19.

 $\geq 30\mu\text{M}$ (20°C) PAL-*chiro*-inositol 2,3,5-trisphosphorothioateL-*chiro*-Ins(2,3,5)PS₃

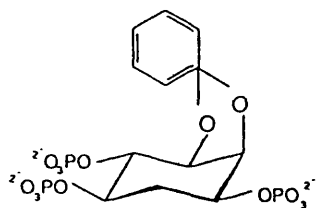
20.

 $\geq 30\mu\text{M}$ (4°C) PAD-*myo*-inositol 1,4,6-trisphosphorothioateIns(1,4,6)PS₃

21.

 $\geq 100\mu\text{M}$ (20°C) InactiveD-*myo*-inositol-6-deoxy 1,4,5-trisphosphate6-deoxy-Ins(1,4,5)P₃

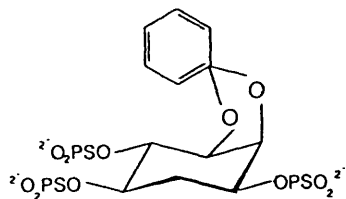
22.

 $\geq 100\mu\text{M}$ (20°C) InactiveD-*myo*-inositol-6-deoxy-2,3-cyclohexyldiene

1,4,5-trisphosphate

6-deoxy-2,3-cyclohex-Ins(1,4,5)P₃

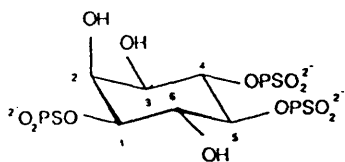
23.

 $\geq 100\mu\text{M}$ (20°C) InactiveD-*myo*-inositol-6-deoxy-2,3-cyclohexylidene

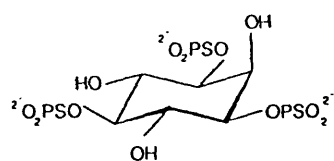
1,4,5-trisphosphorothioate

6-deoxy-2,3-cyclohex-Ins(1,4,5)PS₃

24.

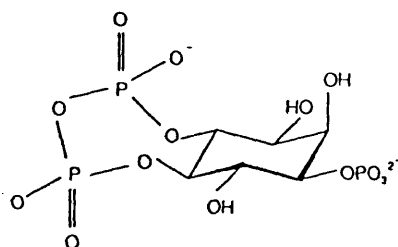
 $\geq 100\mu\text{M}$ (20°C) InactiveL-*myo*-inositol 1,4,5-trisphosphorothioateL-Ins(1,4,5)PS₃

25.

 $\geq 100\mu\text{M}$ (20°C) Inactive

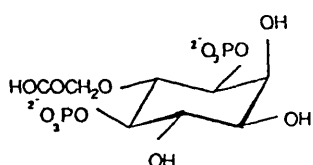
D-*myo*-inositol 1,3,5-trisphosphorothioate
Ins(1,3,5)PS₃

26.

 $\geq 100\mu\text{M}$ (20°C) Inactive

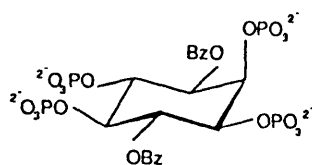
D-*myo*-inositol-1-phosphate 4,5-pyrophosphate
Ins(1)P(4,5)pyrophosphate

27.

 $\geq 100\mu\text{M}$ (20°C) Inactive

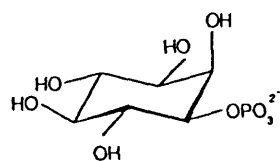
D-*myo*-inositol 4-methylene carboxylate
3,5-bisphosphate
Ins(4)CO(3,5)P₂

28.

 $\geq 100\mu\text{M}$ (20°C) Inactive

D-*myo*-inositol-3,6-dibenzoyl 1,2,4,5-tetrakisphosphate
Ins(3,6)Bz₂(1,2,4,5)P₄

29.

 $\geq 100\mu\text{M}$ (20°C) Inactive

D-*myo*-inositol 1-phosphate
Ins(1)P

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PAPERS ARISING FROM THIS THESIS

Papers

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Abstract

J., Al-Hafidh, Mills, S. J., Lampe, D., Liu, C., J., Potter, B. V. L, and Westwick, J. Myo-inositol-1,4,6-trisphosphorothioate: an inhibitor of inositol trisphosphate-induced $^{45}\text{Ca}^{2+}$ release. Presented at the winter BPS meeting.